REMARKS

I. STATUS OF THE CLAIMS

By this Amendment, claims 5-8 have been cancelled and claims 9 and 16 have been amended as recommended by the Examiner in the Office Action and Interview of March 1, 2005. Support can be found throughout the specification. Thus, claims 9-16 are currently pending. No new matter has been added.

II. INTERVIEW SUMMARY

Applicant thanks Examiner Huang and SPE Tsang for conducting an interview with Applicant's undersigned representative on March 1, 2005. During the interview, all the pending rejections were discussed, as summarized below.

With reference to the rejection under 35 U.S.C. § 102, the Examiner acknowledged that Hirai JP 04-077476 ("Hirai") does not expressly teach inhibiting urease or *H. Pylori* activity. Instead, the Examiner confirmed that Hirai is being relied upon as an allegedly inherent teaching of the claimed methods. The Examiner contended that Hirai inherently anticipates because it states that their compounds can be used to treat ulcers. The Examiner also provided an English language translation of the reference.

Applicant's representative explained that Hirai fails to inherently anticipate because, among other things, treating a NSAID-based ulcer according to Hirai would not inherently inhibit urease or *H. Pylori* activity. SPE Tsang agreed that if a reference

does not teach treating the same patient population subset, it would not inherently anticipate.[1]

The rejections of claim 5-8 under 35 U.S.C. § 112, first paragraph were discussed, but no agreement was reached. The rejections of claim 14 on the grounds of written description and enablement were also discussed. During the interview the Examiner considered the support in the specification for this claim, and indicated that the rejection would be reconsidered in view of further arguments on the record.

With regard to the rejections under 35 U.S.C. § 112, second paragraph, claims 11, 12, and 15 had been rejected for use of the word "comprising." The Examiner explained that, in her view, the use of the term "comprising" rendered these dependent claims somehow broader than parent claim 9. Applicant's representative respectively explained, together with SPE Tsang, that it was not possible for these dependent claims to be broader than their parent, and that the rejection was therefore in error.

Claims 9 and 16 had been rejected for not specifying an amount of the compound that would be administered. The Examiner agreed that the rejection of claims 9 and 16 under Section 112, second paragraph could be overcome by amending the claims to recite "administering to a person in need thereof <u>a therapeutically effective amount of</u> an isothiazole compound. . . ."

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[1] SPE Tsang's explanation is consistent with *Jansen v. Rexall Sundown, Inc.*, holding that a claimed method of treatment is not anticipated unless that reference provides for practicing the method with the intent to achieve the claimed objective. 342 F.3d 1329, 1333-34 (Fed. Cir. 2003) ("In other words, administering the claimed vitamins in the claimed doses for some purpose other than [the claimed] treating or preventing macrocytic-megaloblastic anemia is not practicing the claimed method...."); *Rapoport v. Dement*, 254 F.3d 1053 (Fed. Cir. 2001); *Glaxo Group Ltd. v. Teva Pharma, Inc.,* 2004 U.S. Dist. LEXIS 16750, at *56-57 (D. Del. 2004).

III. REJECTION UNDER 35 U.S.C. § 102 OVER HIRAI

Claims 9-13, 15 and 16 were been rejected over JP 04-077476 ("Hirai"). (Office Action, pg. 3-4.) Applicant respectfully traverses. In particular, Applicant disagrees that Hirai teaches (expressly or inherently) a method of use of Hirai's 1,2-benzoisothiazol-3-(2H)-one for inhibiting urease or as an anti-Helicobacter pylori agent.

As is well known, and as previously evidenced by Applicant on the record, gastrointestinal ulcers can be generally caused by a variety of conditions or factors, including 1) infection with Helicobacter pylori, 2) use of non-steroidal anti-inflammatory drugs: NSAIDs, 3) unusually strong digestive activity with excess secretion of gastric acid, or 4) others (*e.g.*, stress). Thus, all gastrointestinal ulcers are not caused by or associated with Helicobacter pylori infection. For this reason, the treatment of an ulcer generally, or more specifically, for example, an ulcer caused by NSAIDs, would not inherently be treating injury caused by urease or Helicobacter pylori infection, which are not present or associated with all ulcers.

Hirai does not teach (expressly or inherently) or suggest a method for treating gastric mucosa injury caused by urease or anti-Helicobacter pylori activity. In particular, the descriptions on treating ulcer are cited from Hirai as follows:

> 1) "It has been known that 2-[(3,5-dimethyl-4-methoxy-2-pyridyl)methylsulfinil]-5-methoxy-(1H)-benzimidazole (omeprazole) (a compound described in Japan kokai 54-141783) is clinically effective for suppressing gastric acid secretion as antiulcer agent by inhibiting H+, K+-adenosine triphosphatase (H+, K+ -ATP) which is an enzyme relating to the final stage of gastric acid secretion." (See Prior Art on page 4 of the English translation.)
>
> 2) "The inventors made earnest studies on synthesis of benzisothiazolone derivatives and their pharmacological

activity for years, consequently they discovered that the compounds of this invention have an enzymatic inhibition activity of H+, K+-adenosine triphosphatase over 100 times as much as that of above known omeprazole and become an excellent agent for preventing and treating ulcer, thus came to accomplish this invention." (See Problem to be Solved by the Invention on page 4 of the English translation.)

3) "As described above, the compound (I) of this invention have excellent inhibitory action on H+, K+-ATPase and are useful for the prevention or treatment of gastrointestinal diseases, etc., of mankind, e.g., gastric ulcer, duodenal ulcer, or Zollinger-Ellison syndrome, etc." (See page 39 of English translation.)

Hirai simply and only discloses a method of preventing or treating gastrointestinal diseases caused by the above 3) according to administration of the compound (I). However, <u>Hirai is silent about treating gastrointestinal ulcer caused by infection with Helicobacter pylori</u>. Practicing Hirai would not inherently be a treatment method as claimed. Indeed, it is generally considered that Helicobacter pylori is distinct from the ATPase activity of 3) above, and directly injures gastric mucosa or produce inflammatory-causing factor which brings about epithelium cell injuries on gastric mucosa.

For example, according to Huang, J.Q. et al., Lancent, 359:14-22, 2002 ("Huang") (Attachment 1) both Helicobacter pylori infection and NSAID use independently and significantly increase the risk of peptic ulcer and ulcer bleeding (Huang, pg. 14, col. 1, Interpretation.) While having Helicobacter pylori infection and

taking NSAIDs increased the risk of bleeding ulcers,[2] a substantial portion of NSAID patients had ulcers in the absence of Helicobacter pylori infection. (Huang, pg. 14, col. 1, Findings, Interpretation.) Further, the contribution of NSAIDs is significant, since peptic ulcer disease was significantly more common in NSAID takers irrespective of Helicobacter pylori infection. (Huang, pg. 14, col. 1, Findings.) Huang also reports on the controversy of whether eradication of Helicobacter pylori infections retards ulcer healing in NSAID takers. (Huang, pg. 19, col. 2, - page 20, col. 1.)

The fact that use of NSAIDs results in peptic ulcer by a mechanism different from that of Helicobacter pylori infection is further shown in Wolfe, M.M. et al.: N. Engl. J. Med., 340:1888-1899, 1999 (Attachment 2), Wallace, J.L. et al.: Gastroenterology, 119:706-714, 2000 (Attachment 3), and Langenbach, R. et al.: Cell, 83:483-492, 1995(Attachment 4).

An optimal pH for the growth of Helicobacter pylori is 6-7, but this microorganism can survive in the stomach which secretes gastric acid having pH 1-2. This is because gastric acid surrounding a Helicobacter pylori is partially neutralized by ammonia formed from urea in the stomach by the action of Helicobacter pylori secreted urease. The present invention has been completed based on the novel and non-obvious finding that urease activity is remarkably and unexpectedly inhibited by an isothiazole compound according to claimed formula (I).

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[2] The possibility that ulcer may be aggravated by both NSAIDs and Helicobacter pylori does change the fact that ulcers may be due to NSAIDs alone in the absence of Helicobacter pylori infection, and that the treatment of these ulcers is not (even inherently) a treatment of Helicobacter pylori infection or urease activity.

In view of the above, as well as Applicant's previous remarks on the record, there is no support for the Office to maintain that Hirai inherently discloses a method of urease inhibition or anti-Helicobacter pylori activity. It simply does not teach (or even suggest) a method as claimed. Among other things, in the large number of ulcer patients that do not have Helicobacter pylori infection, the use of a compound according to Hirai would not and cannot provide urease inhibition or anti-Helicobacter pylori activity.

Of course, to establish inherency, the evidence "must make clear that the missing descriptive matter is <u>necessarily present</u> in the thing described in the reference ...." <u>Continental Can Co. USA, Inc. v. Monsanto Co.</u>, 20 USPQ2d 1746, 1749 (Fed. Cir. 1991) (emphasis added). It is also well settled that "[i]nherency ... <u>may not</u> be established by probabilities or possibilities. The mere fact that a certain thing may result from a given set of circumstances is not sufficient." <u>Id</u>. (internal citations omitted) (emphasis added). That is, the fact that a certain result or characteristic may occur or be present in the prior art is <u>not sufficient</u> to establish the inherency of that result or characteristic. Therefore, practicing Hirai on a ulcer patient generally, or a NSAID patent as specific example, would not be inherently practicing the claimed method because the patient would not necessarily have a Helicobacter pylori.

For at least the above reasons and those of record, reconsideration and withdrawal of the rejection are respectfully requested.

IV. REJECTIONS OF CLAIMS 5-8 UNDER 35 U.S.C. § 112, FIRST PARAGRAPH

Claims 5-8 and 14 under 35 U.S.C. § 112, first paragraph. In order to advance prosecution, but without conceding to the rejection, claims 5-8 have been cancelled without prejudice or disclaimer.

Concerning claim 14, the Office contends that claim 14 is not enabled. (Office Action of Dec. 23, 2004, pg. 3.) However, in making an enablement rejection, the Office must consider all of the so-called *Wands* factors, which include the state of the prior art and the level of one of ordinary skill. *In re Wands*, 858 F.2d 731, 737 (Fed. Cir. 1988). Because the Office has not addressed, among other things, the skill in the art, the rejection is formally flawed.

Moreover, substantively, the level of skill in the art is particularly high given that treating physicians commonly prescribe multiple active agents. Indeed, as noted in the PDR entry for Prilosec® filed with Applicant's previous Amendment, this proton pump inhibitor (one of the active ingredient classes recited in claim 14) is commonly prescribed with antimicrobials. (*E.g.,* 2004 PDR at pg. 634.) This is evidence that prescribing combined active agents of these classes are within the skill in the art.

Additionally, contrary to the premise of the rejection that the specification lacks sufficient description of the active ingredients, Applicant respectfully directs the Office's attention to, for example, page 15, lines 13-24. The specification thus provides descriptions and examples of active ingredients in the various classes discussed. The specification further explains that "[a]ccording to such combined administration, it is sometimes made possible to eradicate Helicobacter pylori with high probability and to

more easily achieve complete recovery from gastrointestinal diseases caused such as chronic gastritis and gastroduodenal ulcer."

Accordingly, there is no evidence that one skilled in the art would be unable to practice the claimed subject matter without undue experimentation. To the contrary, the level of skill among treating physicians is demonstratably high and the specification provides further guidance to enable the claimed subject matter. Reconsideration and withdrawal of the rejection of claim 14 are respectfully requested.

V. REJECTIONS OF CLAIMS 9-12 AND 14-16 UNDER 35 U.S.C. § 112, SECOND PARAGRAPH

Claims 9-12 and 14-16 were rejected under 35 U.S.C. § 112, second paragraph. (Office Action, pg. 4-5.) Applicant respectfully traverses the rejections.

Claims 11, 12, and 15 were rejected for use of the word "comprising." As noted above, in the Interview of March 1, 2005, the Examiner explained that, in her view, the use of the term "comprising" rendered these dependent claims somehow broader than parent claim 9. Applicant respectfully disagrees, and traverses the rejection.

Claims 9 and 11 (as an example) respectively recite "A method of treating gastric mucosa injury caused by urease" and "A method according to claim 9, wherein the gastric mucosa injury comprises chronic gastritis." Claim 11 necessarily further limits claim 9 because, according to claim 11, the gastric mucosa injury treated by the method of claim 11 must include chronic gastritis. This is not required according to claim 9. The fact that gastric mucosa injury according to claim 11 can include conditions in addition to chronic gastritis does not render it of broader scope than claim 9.

Claims 9 and 16 had been rejected for not specifying an amount of the compound that would be administered. The Examiner agreed that this rejection could be overcome by amending the claims to recite "administering to a person in need thereof a therapeutically effective amount of an isothiazole compound. . . ."

Reconsideration and withdrawal of the rejections are respectfully requested.

VI. CONCLUSION

Applicant respectfully requests that this Amendment under 37 C.F.R. § 1.114 places claims 9-16 in condition for allowance. Applicant submits that the cancellation of claims 5-8 and the amendment of claims 9 and 16 do not raise new issues or necessitate the undertaking of any additional search of the art by the Examiner. Therefore, this Amendment should allow for immediate action by the Examiner.

Please grant any extensions of time required to enter this response and charge any additional required fees to our deposit account 06-0916.

Respectfully submitted,

FINNEGAN, HENDERSON, FARABOW,
GARRETT & DUNNER, L.L.P.

Dated: June 20, 2005

By: ______________________
Mark J. Feldstein
Reg. No. 46,693

Attachments:

1 Huang, J.Q. et al.: Lancent, 359:14-22, 2002

2 Wolfe, M.M. et al.: N. Engl. J. Med., 340:1888-1899, 1999

3 Wallace, J.L. et al.: Gastroenterology, 119:706-714, 2000

4 Langenbach, R. et al.: Cell, 83:483-492, 1995

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Role of *Helicobacter pylori* infection and non-steroidal anti-inflammatory drugs in peptic-ulcer disease: a meta-analysis

Jia-Qing Huang, Subbaramiah Sridhar, Richard H Hunt

Summary

Background The relation between *H pylori* infection and use of non-steroidal anti-inflammatory drugs (NSAIDs) in the pathogenesis of peptic-ulcer disease is controversial. We undertook a meta-analysis to address this issue.

Methods By computer and manually we sought observational studies on the prevalence of peptic-ulcer disease in adult NSAID takers or the prevalence of *H pylori* infection and NSAID use in patients with peptic-ulcer bleeding. Summary odds ratios were calculated from the raw data. Tests for homogeneity were done.

Findings Of 463 citations identified, 25 studies met inclusion criteria. In 16 studies of 1625 NSAID takers, uncomplicated peptic-ulcer disease was significantly more common in patients positive than in those negative for *H pylori* (341/817 [41·7%] vs 209/808 [25·9%]; odds ratio 2·12 [95% CI 1·68–2·67]). In five controlled studies, peptic-ulcer disease was significantly more common in NSAID takers (138/385 [35·8%]) than in controls (23/276 [8·3%]), irrespective of *H pylori* infection. Compared with *H pylori* negative individuals not taking NSAIDs, the risk of ulcer in *H pylori* infected NSAID takers was 61·1 (9·98–373). *H pylori* infection increased the risk of peptic-ulcer disease in NSAID takers 3·53-fold in addition to the risk associated with NSAID use (odds ratio 19·4). Similarly, in the presence of risk of peptic-ulcer disease associated with *H pylori* infection (18·1), use of NSAIDs increased the risk of peptic-ulcer disease 3·55-fold. *H pylori* infection and NSAID use increased the risk of ulcer bleeding 1·79-fold and 4·85-fold, respectively. However, the risk of ulcer bleeding increased to 6·13 when both factors were present.

Interpretation Both *H pylori* infection and NSAID use independently and significantly increase the risk of peptic ulcer and ulcer bleeding. There is synergism for the development of peptic ulcer and ulcer bleeding between *H pylori* infection and NSAID use. Peptic-ulcer disease is rare in *H pylori* negative non-NSAID takers.

Lancet 2002; **359:** 14–22
See Commentary page 3

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Introduction

The relation between infection with *Helicobacter pylori* and use of non-steroidal anti-inflammatory drugs (NSAIDs) in the pathogenesis of peptic-ulcer disease is controversial, because studies examining these two risk factors in this disorder have had conflicting results.[1–4] From conventional thinking, the presence of both these well-established risk factors for peptic-ulcer disease would be expected to increase the risk of the disease. However, this was not the case in several observational studies of patients taking NSAIDs, in which peptic-ulcer disease was less frequently diagnosed when *H pylori* infection was present than in patients without the infection.[5,6] Conflicting results have also been reported from randomised controlled clinical trials on whether eradication of *H pylori* infection retards ulcer healing[7,8] or reduces the risk of developing peptic-ulcer disease in NSAID takers.[9,10]

The discrepancies probably reflect a complex relation between *H pylori* infection and NSAID-associated gastropathy as well as methodological heterogeneity between studies. For example, study populations have differed in terms of NSAID exposure, the controls used for comparison, and the definition of ulcer size.[11–18] Therefore, there are four possible situations for *H pylori* infection and NSAID-associated gastropathy: no interaction, or additive, synergistic, or antagonistic effects between the two risk factors. The aims of this analysis were to review systematically the literature on the relation between *H pylori* infection and NSAID-associated gastropathy; to assess the presence and magnitude of any possible interaction on peptic-ulcer disease between these two risk factors; to examine any possible interaction between the two risk factors with respect to the site of ulcer or ulcer bleeding; and to explore any sources of heterogeneity between the published studies.

Methods

Design and procedures

A computerised literature search was done in the MEDLINE, PubMed, and Cochrane databases for relevant systematic reviews published in any language between 1984 and October 2000, with the following MeSH terms and/or textwords: meta-analysis, systematic review, overview, NSAIDs, and *pylori*. 15 potentially relevant citations were identified. By previously described criteria[19,20] and guidelines for the application of meta-analysis in epidemiological studies,[21] these reports were examined critically by one of the authors (J-QH). No review described a systematic search strategy, and methods to include reviewed articles and assessment of study validity and appropriate statistical analyses were not used. Thus, none of these reviews could be classified as a systematic review or meta-analysis. Our meta-analysis was therefore justified.

The following inclusion criteria were used: observational (cross-sectional, case-control, or cohort) studies investigating the prevalence of peptic-ulcer disease in adult patients taking NSAIDs or the

prevalence of *H pylori* infection and NSAID use in patients with peptic-ulcer bleeding, and the relation between *H pylori* infection and NSAID-associated peptic-ulcer disease; documentation of peptic-ulcer disease or ulcer bleeding was required by endoscopy; and *H pylori* infection had to be confirmed by histology, culture, serology, or urea breath test. Duplicate publications or studies published only in abstract form were excluded. We also excluded studies in which patients had reported recent use (3–4 weeks before study entry) of antibiotics or anti-ulcer drugs or a history of gastric surgery.

For the analysis of ulcer bleeding, in addition to these exclusion criteria, we applied an additional criterion to exclude studies that allowed enrolment of patients with non-ulcer gastrointestinal bleeding or gastric tumours and those receiving corticosteroids or anticoagulants.

The following search terms (both as MeSH terms and as keywords) were used to identify potentially relevant primary studies in the three databases mentioned above, without language restriction: NSAIDs, *pylori*, and ulcer or ulcer bleeding. A search on links to related articles was also done wherever available. A recursive hand-search of the references of all articles reviewed and of the retrieved original studies was done to look for studies not identified by the computer search. The following journals were manually searched for potentially relevant articles from January 1989 to October 2000: *Gastroenterology*, *Gut*, *American Journal of Gastroenterology*, *Alimentary Pharmacology and Therapeutics*, *Digestive Disease Sciences*, *European Journal of Gastroenterology and Hepatology*, *Scandinavian Journal of Gastroenterology*, *Journal of Clinical Gastroenterology*, *The Lancet*, *New England Journal of Medicine*, *Archives of Internal Medicine*, *British Medical Journal*, *JAMA*, *Annals of Internal Medicine*, and *Quarterly Journal of Medicine*.

The title and abstract of all potentially relevant studies were screened for their relevance to the study question before retrieval of full articles. However, full articles were also scrutinised for relevance if the title and abstract were ambiguous.

All searches were made independently by two reviewers (J-QH and SS).

Data were extracted from each study by J-QH and SS by means of a structured spreadsheet. In the case of disagreement, a third reviewer (RHH) was consulted. Major items were the primary question of an individual study, study design, characteristics of case and control populations, major exclusion criteria, definition of ulcer or ulcer bleeding, diagnostic method for ulcer or ulcer bleeding, site of ulcer, definition of NSAID use, type of NSAID, test for *H pylori* infection, total number of cases and controls, percentage of smokers, concurrent treatment, and prevalence of *H pylori* infection and ulcer.

The original investigators were contacted for further information on the site of ulcer or *H pylori* status where necessary.[11,18,22,23]

To assess the validity of each study, the following criteria were applied, modified from the guidelines for reading case-control studies proposed by Lichtenstein and colleagues:[24] an explicit statement of the research question and its relevance to the question of this meta-analysis; the methods for identification of cases and controls and their matching techniques; a clear statement of exclusion criteria for cases and controls; definition of NSAID exposure and peptic ulcer; the methods of data collection; and a description of analytical methods and sample size. To avoid subjective assessments, we did not

Ref	Design	Primary question	NSAID takers	Mean age (years)	Controls	Definition of NSAID use	Ulcer size
11	CC	Effect of *H pylori* on NSAID-related gastropathy	96 RA	63·1	96 dyspeptic patients matched by age and sex	Chronic use	Any size
13	CC	Prevalence of *H pylori* and GI mucosal lesions in NSAID takers	96 IHD	48	50 non-IHD patients matched by age	Daily aspirin >4 weeks	≥0·5 cm
12	Cohort	Interaction between *H pylori* and NSAIDs on GI mucosal damage	38 healthy volunteers	23·5	13 healthy individuals matched by age and sex	Daily use for 4 weeks	≥0·5 cm
18	CC	Mucosal blood flow in NSAID users and effect of *H pylori*	70 RA/OA	54*	17 dyspeptic patients, matched by age and sex†	>4 weeks	≥0·5 cm
14	CC	Relation of *H pylori* to gastric lesions in NSAID takers	85 RA	53	100 non-RA patients matched by age and sex	Chronic use >1 month	Not given
15	CC	Interaction between *H pylori* and NSAIDs on PUD	99 patients for endoscopy	57·5	331 patients, unmatched	Regular use before or within 1 month	≥0·5 cm
16	CC	Interaction between *H pylori* and NSAIDs on GI mucosal damage	76 RA	59	97 patients with abdominal symptoms, unmatched	Chronic use ≥3 months	Not given
17	CC	Interaction between *H pylori* and NSAIDs on gastric mucosa	174 RA	59*	44 RA, non NSAID users, unmatched	>4 weeks	≥0·5 cm
62	CS‡	Interaction between *H pylori* and NSAIDs on PUD	181 RD	61·5	Not available	Chronic use >3 months	≥0·5 cm
58	CS	Interaction between *H pylori* and NSAIDs on GI mucosal damage	128 dyspeptic patients	79·5	Not available	Any time in the 7 days before	Not given
7	CS	*H pylori* eradication on healing PUD in NSAID takers	246 RD	57·5	Not available	Daily use >4 weeks	≥0·5 cm
59	CS	Interaction between *H pylori* and NSAIDs on PUD	82 RD	54	Not available	Chronic use >3 months	≥0·5 cm
60	CS	Interaction between *H pylori* and NSAIDs on PUD	75 RA/OA	21–75§	Not available	Chronic use	≥0·5 cm
5	CS¶	Interaction between *H pylori* and NSAIDs on PUD	50 RA	65*	Not available	Daily use >6 months	≥0·5 cm
61	CS	Relation of *H pylori* to GI mucosal damage	85 RD	54	Not available	>8 months	Not given
63	CS	Relation of *H pylori* to GI mucosal damage	52 RA	52·8	Not available	Not given	Not given

CC=case control; RA=rheumatoid arthritis; GI=gastrointestinal; IHD=ischaemic heart disease; OA=osteoarthritis; PUD=peptic-ulcer disease; CS=cross-sectional; RD=rheumatoid diseases. *Median. †No data on *H pylori* status for controls. ‡Patients were from one group of a previously finished randomised trial. §Range. ¶Designed as case-control, but no endoscopy was done in controls.

Table 1: **Studies examining the relation between *H pylori* infection and NSAID use in patients with uncomplicated peptic-ulcer disease (listed in order of year of publication)**

Study ref	Odds ratio (95% CI)	Variance	Weight of study	Contribution to Q
14	0·74 (0·28–1·96)	0·25	3·98	13·16
18	4·27 (0·97–18·8)	0·57	1·75	0·006
12	21·9 (0·39–1216)	4·20	0·24	0·60
13	18·1 (6·17–53·0)	0·30	3·32	6·37
11	7·59 (3·05–18·9)	0·22	4·61	1·23
All	..	5·54	13·9	21·37

Cochrane Q 21·37, df=4, p<0·001.

Table 2: **Contribution of each study to the Cochrane Q, the statistical test of heterogeneity in a fixed-effects model**

generate an overall quality score, but validity criteria were used to rank studies. For example, a study with a clearly defined control group matched for age, sex, or both would be ranked more highly than one with a poorly matched control group or no controls. Disagreements were resolved by discussion and consensus between the researchers.

The following considerations were applied to determine the combinability of the individual studies for meta-analysis: study design, matching techniques in case-control studies, methods used for measuring outcome, and the biological plausibility. We also took into account the differences between individual results and the summary estimate of the odds ratio and the results of the tests for homogeneity.

Statistical analysis

The following statistical techniques were used to analyse the data, where appropriate. Summary odds ratio and 95% CI were calculated from the raw data of the selected studies by the method of DerSimonian and Laird in a random-effects model. The Breslow-Day method was used to test for homogeneity under the null hypothesis that the odds ratios were consistent across the selected studies. However, in the presence of statistical heterogeneity, we searched for the sources of any possible clinically important heterogeneity (ie, methodological or biological heterogeneity). We did not simply exclude outliers on the basis of the statistical test of homogeneity, because heterogeneity is expected rather than the exception in meta-analysis in epidemiological studies.[25,26]

Subgroup or sensitivity analyses under a random-effects model were carried out where appropriate.

The measurement of agreement between observers was expressed as the κ coefficient (PC agree). All other statistical analyses were done with EasyMa Software for Meta-analysis (EasyMa 2000).

Results

Publications

The literature search in the three databases generated 463 citations, and screening of citation titles and abstracts identified 61 potentially relevant studies for full article retrieval. Of these, 36 studies were subsequently excluded: *H pylori* eradication study;[8-10] treatment trial with omeprazole or H_2-receptor antagonists or misoprostol;[27-29] no baseline endoscopy data;[30] no raw data on peptic-ulcer disease or *H pylori* status;[31-50] inclusion of patients negative for *H pylori* only;[51] inclusion of patients recently exposed to H_2-receptor antagonists;[52,53] all patients had peptic-ulcer disease;[54,55] no contemporary control in patients with bleeding peptic-ulcer disease;[22] patients with ulcer perforation;[56] and review article.[57]

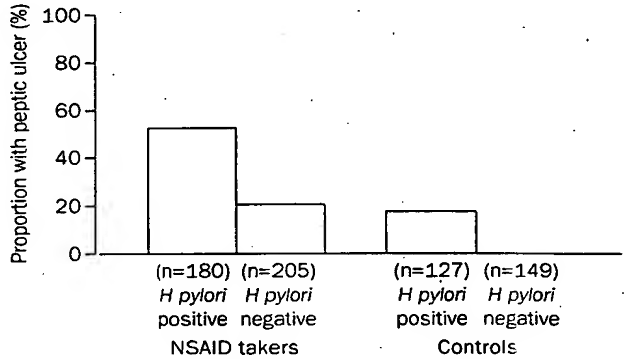

Figure 1: **Prevalence of peptic-ulcer disease among NSAID takers and controls by *H pylori* status**

Manual search of the references of the retrieved articles and major relevant medical journals did not yield any new studies.

The inter-observer agreement on study selection was high (κ=0·946).

Uncomplicated peptic-ulcer disease

16 studies provided raw data on the prevalence of peptic-ulcer disease in 1633 NSAID takers (table 1).[5,7,11-18,58-63] However, data on *H pylori* status were available for only 1625 patients. The pooled frequency of peptic-ulcer disease was 41·7% (341/817) in *H pylori* positive NSAID takers and 25·9% (209/808) in NSAID takers negative for *H pylori*, which gives a summary odds ratio of 2·12 (95% CI 1·68–2·67; Breslow-Day test p=0·43). Similar results were found when studies were grouped by study design, with a summary odds ratio of 3·53 (2·16–5·75) for controlled studies with matching for age, sex, or both, and 1·83 (1·41–2·38) for non-controlled studies.

Because individual patients' data were not available, analysis of age-adjusted prevalence of *H pylori* infection was not possible. However, plotting of study-specific odds ratios against mean or median age in each study did not identify any association between age and study findings (Pearson correlation coefficient –0·402, p=0·138).

Eight controlled studies compared the frequency of uncomplicated peptic-ulcer disease in NSAID takers and

Study ref	PUD in NSAID takers			PUD in non-NSAID takers		
	Number of cases/total		Odds ratio (95% CI)	Number of cases/total		Odds ratio (95% CI)
	H pylori positive	*H pylori* negative		*H pylori* positive	*H pylori* negative	
14	3/26	4/59	1·79 (0·37–8·66)	11/59	0/41	38·5 (0·72–2071)
18	17/30	10/40	3·92 (1·42–10·9)	2/13	0/4	3·40 (0·05–246)
12	9/24	2/14	3·60 (0·65–19·9)	0/8	0/5	0·64 (0·002–181)
13	49/65	11/31	5·57 (2·20–14·1)	4/13	0/37	68·5 (1·14–4124)
11	17/35	16/61	2·66 (1·11–6·37)	6/34	0/62	55·1 (0·99–3078)
Summary			3·53 (2·16–5·75)			18·1 (2·64–124)

PUD=peptic-ulcer disease. Breslow-Day test for homogeneity gave p=0·72 for PUD in NSAID takers, and p=0·32 for that in non-NSAID takers.

Table 3: **Analysis of studies with controls matched for age and sex by *H pylori* status**

Study ref	PUD in NSAID takers			PUD in non-NSAID takers		
	Cases/total		Odds ratio (95% CI)	Cases/total		Odds ratio (95% CI)
	H pylori positive	H pylori negative		H pylori positive	H pylori negative	
18	17/30	10/40	3·92 (1·42–10·9)	2/13	0/4	3·40 (0·05–246)
12	9/24	2/14	3·60 (0·65–19·9)	0/8	0/5	0·64 (0·002–181)
13	49/65	11/31	5·57 (2·20–14·1)	4/13	0/37	68·5 (1·14–4124)
11	17/35	16/61	2·66 (1·11–6·37)	6/34	0/62	55·1 (0·99–3078)

PUD=peptic ulcer disease. Breslow-Day test for homogeneity gave p=0·73 for PUD in NSAID takers and p=0·25 in non-NSAID takers. Odds ratio for *H pylori* positive NSAID takers *vs H pylori* negative NSAID takers 3·79 (2·27–6·34); for *H pylori* positive NSAID takers *vs H pylori* positive non-NSAID takers 5·62 (2·73–11·6); for *H pylori* positive NSAID takers *vs H pylori* negative non-NSAID takers 77·3 (10·3–581); for *H pylori* negative NSAID takers *vs H pylori* negative non-NSAID takers 21·6 (2·82–166); and for *H pylori* positive non-NSAID takers *vs H pylori* negative non-NSAID takers 14·4 (1·60–129).

Table 4: **Sensitivity analysis of controlled studies with matching for age, sex, or both, by *H pylori* status**

non-NSAID takers (table 1).[11-18] NSAID takers and controls were not matched by age in three of these studies.[15-17] Because *H pylori* infection is age dependent, we therefore analysed the prevalence of *H pylori* infection only in the remaining five studies.[11-14,18] In the study by Kordecki and colleagues,[13] two age-matched control groups were used for comparison with the NSAID takers. One consisted of patients with a similar primary diagnosis of disease to the NSAID takers, but with a history of peptic-ulcer disease. Furthermore, these controls might have received anti-ulcer treatment before study entry and cannot be considered as true controls. The other control group consisted of patients about to undergo surgery who had no history of peptic-ulcer disease. For our analysis, the latter control group was used.

Overall, *H pylori* infection was diagnosed in 46·8% (180/385) of the NSAID takers and 46·0% (127/276) of the controls. There was no significant difference in the pooled prevalence of the infection between the two groups (summary odds ratio 0·88 [95% CI 0·28–2·79], Breslow-Day test p<0·001). However, peptic-ulcer disease was significantly more common in NSAID takers than in controls (138/385 [35·8%] *vs* 23/276 [8·3%]; summary odds ratio 5·14 [1·35–19·6]; Breslow-Day test p<0·001) irrespective of *H pylori* infection.

Table 2 shows study-specific odds ratios of the five controlled studies[11-14,18] in a fixed-effects model, and the contribution of each study to the test of heterogeneity. The results show that the heterogeneity was caused predominantly by Caselli and colleagues' study,[14] because no significant heterogeneity was found after exclusion of that study (Breslow-Day test p=0·39).

In the study by Caselli and colleagues,[14] the exclusion criteria were not provided for the selection of controls. We could not be sure, therefore, whether patients with a history of peptic-ulcer disease were included in the control group. A sensitivity analysis that excluded this study gave a summary odds ratio of 9·41 (5·05–17·5).

Figure 1 illustrates the prevalence of peptic-ulcer disease in NSAID takers and controls by *H pylori* status.

The risk of peptic-ulcer disease associated with *H pylori* infection, without NSAID exposure, was calculated by comparing the difference in the frequency of peptic-ulcer disease between controls who were positive and negative for *H pylori* (odds ratio 18·1 [2·64–124]; table 3).[11-14,18]

The risk of peptic-ulcer disease associated with NSAID use, without *H pylori* infection, was estimated by comparing the difference in the prevalence of peptic-ulcer disease between *H pylori* negative NSAID takers and *H pylori* negative controls (odds ratio 19·4 [3·14–120]).[11-14,18]

In the presence of *H pylori* infection, the use of NSAIDs increased the risk of peptic-ulcer disease 3·55-fold (1·26–9·96). Similarly, in the presence of NSAID exposure, *H pylori* infection increased the risk of peptic-ulcer disease 3·53-fold (2·16–5·75; table 3).[11-14,18] However, when the comparison was between NSAID takers with *H pylori* infection and controls without the infection, the risk of peptic-ulcer disease increased to 61·1 (9·98–373).

Table 4 shows the results of sensitivity analysis with Caselli and colleagues' study excluded.

Among the five controlled studies with matching for age, sex, or both, four studies provided data on the site of the ulcer.[11,12,14,18] Table 5 gives the frequency of gastric and duodenal ulcer by *H pylori* status in the four studies and the estimated risk of developing a gastric or duodenal ulcer.

Bleeding peptic-ulcer disease

The literature search identified nine case-control studies assessing the prevalence of *H pylori* infection and NSAID use in 893 patients with bleeding peptic ulcer and 1002 controls without bleeding (table 6).[23,64-71]

Overall, the prevalence of *H pylori* infection was 73·6% (657/893) in the cases and 67·3% (674/1002) in the controls, yielding a summary odds ratio of 1·67 (95% CI 1·02–2·72; Breslow-Day, p<0·001; figure 2). Histology, rapid urease test, and culture have shown significantly higher false-negative rates than serology for diagnosis of *H pylori* infection in patients with bleeding peptic ulcers.[72,73] We therefore undertook subgroup

Study ref	Gastric ulcer				Duodenal ulcer			
	NSAID takers: cases/total		Non-NSAID takers: cases/total		NSAID takers: cases/total		Non-NSAID takers: cases/total	
	H pylori positive	H pylori negative	H pylori positive	H pylori negative	H pylori positive	H pylori negative	H pylori positive	H pylori negative
14	1/26 (4%)	4/59 (7%)	2/59 (3%)	0/41	2/26 (8%)	0/59	9/59 (15%)	0/41
11	13/35 (37%)	16/61 (26%)	3/34 (9%)	0/62	4/35 (11%)	3/61 (5%)	3/34 (9%)	0/62
12	3/24 (13%)	2/14 (14%)	0/8	0/5	6/24 (25%)	0/14	0/8	0/5
18	10/30 (33%)	5/40 (13%)	0/13	0/4	7/30 (23%)	5/40 (13%)	2/13 (15%)	0/4
Odds ratio	1·72		4·07		2·77		9·14	
(95% CI)	(0·92–3·20)		(0·39–42·9)		(1·12–6·88)		(1·02–81·8)	

Breslow-Day test for homogeneity gave p=0·4 for gastric ulcer in NSAID takers; p=0·41 for gastric ulcer in non-NSAID-takers; p=0·42 for duodenal ulcer in NSAID takers; and p=0·51 for duodenal ulcer in non-NSAID-takers.

Table 5: **Analysis of effects of *H pylori* infection and NSAID use on the site of peptic ulcer**

Study ref	Primary question	Study design	Cases and diagnosis of GI bleeding	Mean age (years)	Controls	NSAID use
64	Interaction between *H. pylori* and NSAIDs in PU bleeding	CC	185 patients with bleeding PU verified by clinical and endoscopic findings	55	185 hospital controls, matched by age and sex	<1 week before entry
23	Role of *H pylori* and NSAIDs in PU bleeding	CC	97 patients with haematemesis or melaena 1 week before entry and with endoscopic stigma of recent bleeding	66	97 patients undergoing endoscopy without PU, matched by age and sex	<7 days of endoscopy
65	Role of *H pylori* in NSAID associated GI bleeding	CC	73 patients with haematemesis, melaena or anaemia with a loss of >3 g/dL haemoglobin and with endoscopic stigma of recent bleeding	80	73 non-bleeding patients, matched by endoscopic diagnosis, age and sex	<7 days of endoscopy
66	Interaction between *H pylori* and NSAIDs in upper GI bleeding	CC	72 patients with endoscopically verified PU bleeding	69	72 non-GI patients, matched by age, sex, and race	<1 week before entry
67	Role of *H pylori* in PU bleeding in NSAID users	CC	132 NSAID users with endoscopically verified GI bleeding from PU or haemorrhagic gastritis	72*	136 NSAID users with no sign of GI bleeding, matched by age and sex	<1 week before entry
68	Interaction between *H pylori* and NSAIDs in PU bleeding	CC	82 patients with PU bleeding verified by endoscopy	75	93 hospital controls, matched by age and sex	Not given
69	Role of *H pylori* and NSAID use in GU bleeding	CC	100 patients with GU bleeding verified by clinical and endoscopic findings	67	117 patients with non-bleeding GU, matched by sex	Within 4 weeks before entry
70	Prevalence of *H pylori* and relation to NSAIDs in PU bleeding	CC	106 patients with haematemesis or melaena from PU verified by endoscopy	68	30 healthy volunteers, matched by region and socioeconomic status	<1 week before entry
71	Prevalence of *H pylori* and NSAID use in PU bleeding	CC	46 patients with endoscopically diagnosed PU bleeding	23–83†	199 patients with non-bleeding PU, matching data unknown	1 month before entry

GI=gastrointestinal; PU=peptic ulcer; CC=case-control; GU=gastric ulcer; *Median. †Range.

Table 6: **Studies examining the relation between *H pylori* infection and NSAID use in patients with peptic-ulcer bleeding**

analyses according to *H pylori* testing methods. The summary odds ratio was estimated at 2·16 (1·54–3·04) for studies that used serology (figure 3).[66–68,70] The test of homogeneity was not significant for these studies (Breslow-Day p=0·32). The summary odds ratio for studies that used non-serological tests was 1·24 (0·50–3·08), with a highly significant test of homogeneity (Breslow-Day p<0·001).[23,64,65,69,71]

Seven of the nine studies provided comparable data on the prevalence of NSAID use in cases and controls.[23,64–66,68,69,71] The prevalence of NSAID use was 59·7% (391/655) in the cases and 27·4% (230/839) in the controls, giving a summary odds ratio of 4·79 (3·78–6·06; Breslow-Day p=0·3).

Of the nine studies, six had controls matched for age, sex, or both.[23,64–68] The pooled prevalence of *H pylori* infection in these studies was 70·2% (450/641) in the cases and 56·1% (368/656) in the controls, yielding a summary odds ratio of 1·79 (0·97–3·32; Breslow-Day p<0·001), irrespective of the method of testing for *H pylori* infection. In subgroup analyses on *H pylori* testing methods, the summary odds ratio for studies that used serology was 2·13 (1·38–3·31; Breslow-Day p=0·19).[66–68] For studies that used non-serological tests, the summary odds ratio was 1·42 (0·38–5·28; Breslow-Day p<0·001).[23,64,65]

Of the six case-control studies,[23,64–68] the pooled prevalence of NSAID use was 58·6% (357/609) in the cases and 23·5% (150/637) in the controls, giving a summary odds ratio of 4·85 (3·77–6·23; Breslow-Day p=0·21).

In the comparison between *H pylori* infected NSAID takers (64·5%, 149/231) and *H pylori* negative controls not taking NSAID (23·0%, 40/174), the risk of developing ulcer bleeding was 6·13 (3·93–9·56).[23,64] This value is almost the sum of the two odds ratios estimated for *H pylori* infection (1·79) and NSAID use (4·85).

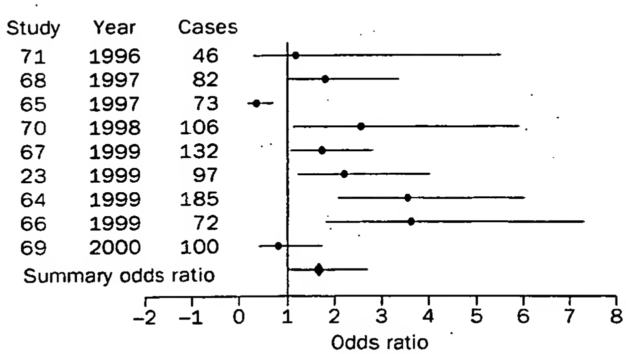

Figure 2: **Study-specific and summary odds ratios in all case-control studies assessing the prevalence of *H pylori* infection in patients with bleeding ulcers**
Odds ratio is represented by oval or diamond symbol; 95% CI by horizontal lines.

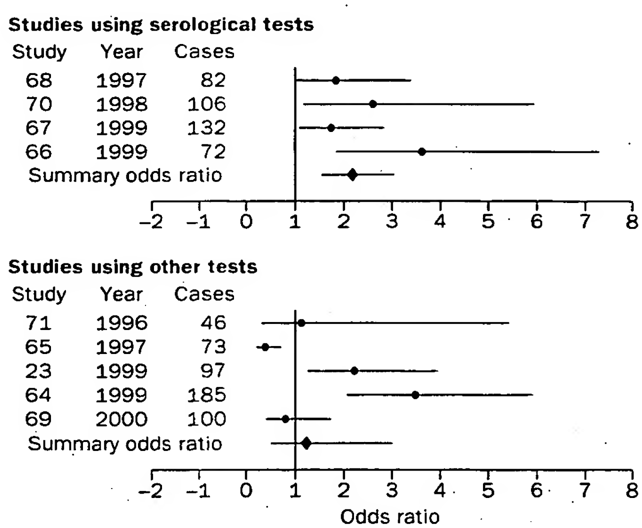

Figure 3: **Study-specific and summary odds ratios in studies using serology or other tests for *H pylori* infection**

Discussion

Previous studies have shown that *H pylori* infection or use of NSAIDs confers a three-fold to four-fold increased risk of peptic-ulcer disease.[74–76] However, whether the magnitude of the risk reported in these studies was for the individual or combined contribution of *H pylori* infection and NSAIDs to the development of peptic-ulcer disease or ulcer complications was not known.

We have found, in this systematic review, that a third of patients taking NSAIDs long term had gastric or duodenal ulcers, irrespective of *H pylori* status and study design. However, peptic-ulcer disease was significantly more common in *H pylori* infected NSAID takers than in takers without the infection, suggesting a possible interaction between NSAID use and *H pylori* infection for the development of peptic ulcer.

Because the prevalence of *H pylori* infection is significantly age dependent, assessment of the effect of *H pylori* infection on NSAID-associated peptic-ulcer disease in age-matched controlled studies is meaningful. In studies for which an age-matched control group was available, NSAID use increased the risk of peptic ulcer five-fold compared with non-NSAID takers, irrespective of *H pylori* status. However, *H pylori* infection increased the risk of peptic ulcer disease 3·5-fold and 18-fold in NSAID takers and controls, respectively. The odds ratio of 3·5 among NSAID takers is explained by the increased risk of peptic-ulcer disease in the presence of *H pylori* infection in addition to the risk associated with NSAID use (odds ratio 19·4). The extremely large odds ratios seen in the comparisons between control populations might have resulted from a zero event rate in *H pylori* negative controls, because statistical modelling by adding a constant pseudo-count of 1 to the analysis yielded a more meaningful and reliable odds ratio (6·36 [95% CI 2·21–18·3]). Nevertheless, the results are consistent with clinical and epidemiological data that peptic-ulcer disease is related primarily to *H pylori* infection and NSAID use.[77]

No peptic-ulcer disease was seen in patients without *H pylori* infection who were not taking NSAIDs in the five controlled studies (figure 1).[11–14,18] Therefore, this population is the true control population for assessment of any possible interaction between *H pylori* infection and NSAID use for the development of peptic-ulcer disease. As shown in this analysis, the effect of *H pylori* infection and NSAID use on peptic-ulcer disease, as shown by the magnitude of risk, was additive when *H pylori* infected NSAID takers were compared with the true controls; the results were confirmed by sensitivity analyses, which suggests a synergism for the development of peptic-ulcer disease between these two risk factors.

Another uncertainty is whether, in NSAID takers, *H pylori* infection is an important risk factor for gastric ulcer as it is for duodenal ulcer, on the basis of the existing evidence.[5,7,11–18,58–63] Most published studies did not separate patients by the location of ulcer and reported these ulcers together.[5,7,13,15–17,58–63] Our pooled analysis of four studies showed that *H pylori* infection is less closely associated with gastric ulcer than with duodenal ulcer in both NSAID takers and control groups,[11,12,14,18] although this result might have been caused by a small sample size. Nevertheless, the results suggest that NSAID use has a major role in the development of gastric ulcer, whereas duodenal ulcer is more closely related to *H pylori* infection. The mixed patient population may have contributed to the conflicting published results. Therefore, future studies should separate clearly patients with gastric ulcer from those with duodenal ulcer when examining the relation between *H pylori* infection and NSAID-associated peptic-ulcer disease.

There has been debate over the clinical importance of endoscopically detected gastric and duodenal ulcers associated with NSAID use because of a weak relation between endoscopically observed gastroduodenal mucosal lesions and symptoms.[78,79] However, previous studies have not taken *H pylori* infection into account.[76] Therefore, whether there is any relation between endoscopically observed ulcers and symptoms in patients infected with *H pylori* is not known. Thus, results from studies of patients with ulcer complications may provide more important and clinically relevant information on the interaction between *H pylori* infection and NSAID use. In this systematic review, we found that NSAID use (odds ratio 4·85) was significantly more common in patients with bleeding peptic ulcer than in controls, whereas *H pylori* infection (1·79) only marginally increased the risk of ulcer bleeding. However, when both risk factors coexist, the magnitude of the risk was additive (6·13), which suggests that both *H pylori* infection and NSAID use contribute to peptic-ulcer bleeding with NSAIDs having a major role, on the basis of the magnitude of the risk ratio.

The recent debate over the role of *H pylori* infection in NSAID-associated peptic-ulcer disease has been fuelled largely by the conflicting results from two randomised controlled clinical trials.[9,10] The differences between these two studies have been extensively reviewed elsewhere.[80,81] The panel summarises major differences in the methodology and findings. These differences are fundamental and may help to explain the contrasting conclusions from these two studies.

Whether eradication of *H pylori* infection retards ulcer healing in NSAID takers is also controversial.[7,8] Chan and colleagues reported, in a randomised clinical trial of 195 *H pylori* infected patients with NSAID-associated bleeding ulcer, that eradication of the infection did not impair ulcer healing compared with antisecretory treatment alone.[8] By contrast, Bianchi Porro and colleagues found that ulcer healing rate was reduced by *H pylori* eradication, although successful eradication of

Major differences between studies by Chan and colleagues[9] and Hawkey and colleagues[10]

Features	Chan et al[9]	Hawkey et al[10]
Population	Chinese	European
Long-term NSAID use	Excluded	Included
Ulcer history	Excluded	Included (6% more ulcer at entry in the eradication group)
Definition of ulcer	≥5 mm	≥3 mm
NSAID used	Naproxen	Various
Eradication regimen	1 week bismuth triple	1 week omeprazole triple
Follow-up period	2 months	6 months
Ulcer by randomisation		
Eradication	7%	44%
Controls	26%	47%
Ulcer by final *H pylori* status		
H pylori eradicated	2·5%	Not provided
H pylori persisted	26%	Not provided

the infection decreased ulcer recurrence by 15% during 6 months of follow-up compared with patients with persistent infection.[7] More recently, Chan and colleagues reported that *H pylori* eradication was as effective as omeprazole maintenance treatment for preventing ulcer rebleeding in users of low-dose aspirin, but not in patients taking naproxen,[82] which suggests that *H pylori* eradication cannot replace maintenance treatment with antisecretory agents in regular NSAID takers at high risk of ulcer bleeding.

The results of secondary analyses of two large cohort studies, ASTRONAUT and OMNIUM, are also difficult to interpret and compare with the findings of our analysis because of study design and the primary question of continuous maintenance treatment with antisecretory agents or misoprostol in long-term NSAID takers.[27,29] *H pylori* infection is known to increase the antisecretory effect of omeprazole,[83,84] which might partly explain the difference in ulcer remission between *H pylori* positive and negative patients.[80,81]

In this study, we identified several sources of heterogeneity through sensitivity and subgroup analyses, which might help explain the conflicting results and opinions previously published. For example, in the comparison of uncomplicated peptic-ulcer disease, unclear selection criteria for the control population used in Caselli and colleagues' study[14] accounted for more than half of the heterogeneity among the five controlled studies. In studies examining the relation between *H pylori* infection and NSAID use in ulcer bleeding, different testing methods for *H pylori* infection led to the heterogeneity in the overall and subgroup analyses.

There are likely to be other sources of heterogeneity that have not been identified in this analysis. There are also several limitations. For instance, the prevalence of *H pylori* infection could not be adjusted by age because of the lack of individual patients' data; NSAID takers had various underlying disorders, and different control populations were used. Furthermore, patients could have been exposed to different NSAIDs or aspirin with varying ulcerogenic potency. Different definitions of ulcer could also be a source of bias. Finally, the conclusions drawn from subgroup analyses might be limited by small sample sizes. Nevertheless, we believe that meta-analysis is a useful tool for systematically assessing the totality of evidence and to provide directions for future studies. The results of this analysis may provide grounds for a biologically meaningful argument that the interaction between *H pylori* infection and NSAID use in peptic-ulcer disease is consistent across different populations of patients and study designs.

In conclusion, *H pylori* infection and NSAID use independently increase the risk of peptic-ulcer disease and ulcer bleeding. There is synergism for the formation of peptic ulcer and ulcer bleeding between these two risk factors. Peptic-ulcer disease is rare in *H pylori* negative non-NSAID takers.

Contributors

Jia-Qing Huang and Richard Hunt initiated the project, contacted the original investigators for raw data, and wrote the article; Jia-Qing Huang and Subbaramiah Sridhar did the literature search, data extraction, and validity assessment. Jia-Qing Huang did all the statistical analyses; Richard Hunt was consulted on all issues and discrepancies during the process of the study.

Conflict of interest statement

None declared.

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Uses of error

Learning experiences

Chhanda Bewtra

The year was 1979. I had just finished my residency training and joined my department as a brand new junior pathologist full of enthusiasm and brimming with self-confidence. I was taught by the old masters, who proclaimed diagnostic pathology as the last word, with no room for doubt or error. Reality was quite different.

A 79-year-old heavy smoker presented with haemoptysis, chest pain, and a non-resolving large central lung mass. A bronchoscopy yielded large, atypical cells which I diagnosed as non-small-cell carcinoma and next day the entire lobe with the mass was resected. As I opened it up, I was aghast to see a large, organising infarct. The bronchial tree was clean. I couldn't see any tumours anywhere in the specimen. I submitted over 100 sections to be sure I was not missing something, but it remained stubbornly a benign infarct. I remember the first shock and heart-stopping fear when I realised that I had made a major mistake. Then the desperation, a meticulous search all over the specimen for a non-existent tumour, followed by intense fear that my budding career was doomed. Then shame, and finally a lack of self-confidence that dogged me for weeks. I showed all the slides to my colleagues. Everyone agreed, it was an error, a false-positive diagnosis of cancer. I had notified the clinician immediately. He was an experienced pulmonologist, and was not very perturbed. A large, non-resolving infarct like this needed to be resected. "No harm done", he said patronisingly, but I was so mortified that for a week I couldn't even visit the doctors' lounge, fearing everyone was talking about my shameful error. Obviously, as a neophyte, I was very self-conscious. I remember having an intense desire to go to the patient's room and apologise for my error. I decided this was not a good idea in such a litigious society. I still wish there were a safe outlet for physicians to acknowledge their errors.

The patient recovered uneventfully. I did a literature search and found only one report of a pulmonary infarct with atypical cytology. I wrote up a modest research proposal to study this phenomemon, first in a canine model and later in a prospective human study. Fortunately, this study earned me my first research grant and numerous publications describing this dangerous pitfall in the diagnosis of lung cancer. I learnt a lesson here, and by subsequent research and publications I have tried to educate my peers about my mistake. I still have the slides and use them to teach medical students and residents about errors and how to handle them. Looking back, I smile ruefully at my intense reaction.

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Review Article

Medical Progress

Gastrointestinal Toxicity of Nonsteroidal Antiinflammatory Drugs

M. Michael Wolfe, M.D., David R. Lichtenstein, M.D., and Gurkirpal Singh, M.D.

ONE hundred years have passed since Felix Hoffman, working at Bayer Industries, reported the successful synthesis of acetylsalicylic acid as the first nonsteroidal antiinflammatory drug (NSAID).[1,2] At the suggestion of Hermann Dreser, Bayer's chief pharmacologist at the time,[3] the compound was called "aspirin" and was purported to represent a convenient mechanism for the delivery of salicylic acid in the treatment of rheumatic diseases, menstrual pain, and fever.[2] Approximately 40 years elapsed before Douthwaite and Lintott[4] provided endoscopic evidence that aspirin could cause gastric mucosal damage. Numerous reports have corroborated this observation,[5-8] and the introduction of more potent agents with an even greater propensity for toxic effects has increased the awareness of NSAID-induced gastroduodenal ulcer and provided impetus for the development of effective NSAIDs with a more favorable safety profile.

Starting in the early 1970s, numerous new NSAIDs were developed that were initially believed to be devoid of gastrointestinal toxicity, but few, if any, are entirely harmless. These agents constitute one of the most widely used classes of drugs, with more than 70 million prescriptions and more than 30 billion over-the-counter tablets sold annually in the United States.[9] Although NSAIDs are generally well tolerated, adverse gastrointestinal events occur in a small but important percentage of patients, resulting in substantial morbidity and mortality.

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EPIDEMIOLOGY OF GASTROINTESTINAL COMPLICATIONS

Because of the broad and nonspecific definitions of gastrointestinal disorders caused by the use of NSAIDs, as well as differences in patient populations, drugs, dosages, and periods of use, estimates of the prevalence of adverse effects vary greatly. In general, at least 10 to 20 percent of patients have dyspepsia while taking an NSAID, although the prevalence may range from 5 to 50 percent.[10,11] Within a six-month period of treatment, 5 to 15 percent of patients with rheumatoid arthritis can be expected to discontinue NSAID therapy because of dyspepsia.[11]

According to prospective data from the Arthritis, Rheumatism, and Aging Medical Information System (ARAMIS), 13 of every 1000 patients with rheumatoid arthritis who take NSAIDs for one year have a serious gastrointestinal complication. The risk in patients with osteoarthritis is somewhat lower (7.3 per 1000 patients per year).[12]

The rate of NSAID-related serious gastrointestinal complications requiring hospitalization has decreased in recent years. The decrease may be due, at least in part, to extensive medical-education campaigns that have persuaded physicians to use newer, less toxic NSAIDs and non-NSAID analgesics in populations at high risk.[12]

The mortality rate among patients who are hospitalized for NSAID-induced upper gastrointestinal bleeding is about 5 to 10 percent.[13] An analysis of data from ARAMIS has shown that the mortality rate attributed to NSAID-related gastrointestinal toxic effects is 0.22 percent per year, with an annual relative risk of 4.21 as compared with the risk for persons not using NSAIDs.[12] Although the annual mortality rate is low, it must be emphasized that because a large number of patients are exposed to NSAIDs, often for extended periods of time, the risk over a lifetime is substantial. In the United States, for instance, it is estimated that NSAIDs are used regularly by at least 13 million people with various arthritides. On the basis of these conservative figures and ARAMIS data, the annual number of hospitalizations in the United States for serious gastrointestinal complications is estimated to be at least 103,000. At an estimated cost of $15,000 to $20,000 per hospitalization, the annual direct costs of such complications exceed $2 billion.[14]

It has been estimated conservatively that 16,500 NSAID-related deaths occur among patients with rheumatoid arthritis or osteoarthritis every year in the United States. This figure is similar to the number of deaths from the acquired immunodeficiency

syndrome and considerably greater than the number of deaths from multiple myeloma, asthma, cervical cancer, or Hodgkin's disease (Fig. 1).[12,15] If deaths from gastrointestinal toxic effects of NSAIDs were tabulated separately in the National Vital Statistics reports, these effects would constitute the 15th most common cause of death in the United States. Yet these toxic effects remain largely a "silent epidemic," with many physicians and most patients unaware of the magnitude of the problem.[12] Furthermore, the mortality statistics do not include deaths ascribed to the use of over-the-counter NSAIDs.

In a recent survey of 4799 Americans, 807 were identified who had taken NSAIDs (prescribed or over-the-counter drugs) at least twice in the past year for five or more consecutive days.[12] Approximately 45 percent of the group took NSAIDs for five or more consecutive days at least once per month, and 40 percent took both over-the-counter and prescribed NSAIDs. Nearly 75 percent of those who used NSAIDs regularly were either unaware of or unconcerned about possible gastrointestinal complications. In addition, almost two thirds of the regular users indicated that they would expect warning signs before the development of serious NSAID-induced complications. Only a minority of patients who have serious gastrointestinal complications report any antecedent dyspepsia.[11,13] In a study of patients with serious gastrointestinal complications, Singh et al.[11] found that although the proportion of patients with prior symptoms was only slightly higher than the proportion with no prior symptoms (2.7 percent vs. 2.0 percent), 81 percent of the patients reported no antecedent dyspepsia.

RISK FACTORS FOR GASTROINTESTINAL COMPLICATIONS

Because dyspeptic symptoms are not a reliable warning sign, it is important to identify factors that increase the risk of serious gastrointestinal complications and to determine methods for reducing this risk. A number of studies have been designed to identify patients who are most likely to have adverse effects of NSAID therapy (Table 1).

Advanced age has been consistently found to be a primary risk factor for adverse gastrointestinal events. The risk increases linearly with age.[15-20] Although previous reports suggested that the risk diminishes over time, a recent study indicates that the risk of NSAID-associated gastrointestinal hemorrhage remains constant over an extended period of observation.[12] Other risk factors that have been identified in multiple studies are higher doses of NSAIDs (including the use of two or more NSAIDs), a history of gastroduodenal ulcer or gastrointestinal bleeding, concomitant use of corticosteroids, serious coexisting conditions, and concomitant use of anticoagulants.[20-27] However, many of these studies are based

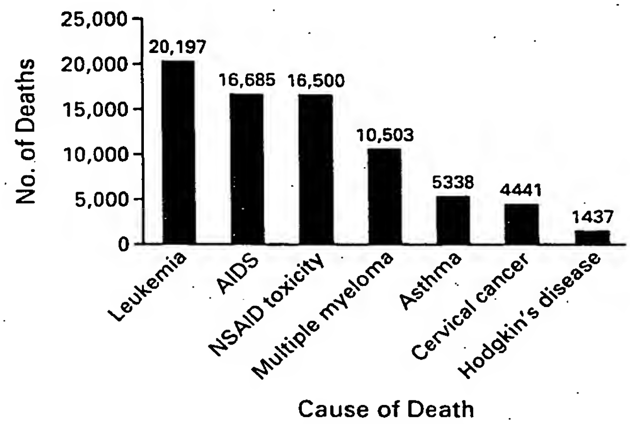

Figure 1. U.S. Mortality Data for Seven Selected Disorders in 1997.
A total of 16,500 patients with rheumatoid arthritis or osteoarthritis died from the gastrointestinal toxic effects of NSAIDs. Data are from the National Center for Health Statistics and the Arthritis, Rheumatism, and Aging Medical Information System.[12]

TABLE 1. RISK FACTORS FOR THE DEVELOPMENT OF NSAID-ASSOCIATED GASTRODUODENAL ULCERS.*

Established risk factors
Advanced age (linear increase in risk)
History of ulcer
Concomitant use of corticosteroids
Higher doses of NSAIDs, including the use of more than one NSAID
Concomitant administration of anticoagulants
Serious systemic disorder
Possible risk factors
Concomitant infection with *Helicobacter pylori*
Cigarette smoking
Consumption of alcohol

*Information on risk factors is from Singh and Triadafilopoulus,[12] Bjorkman,[16] Longstreth,[17] Greene and Winickoff,[18] Gabriel et al.,[19] Griffin et al.,[20] Langman et al.,[21] Garcia Rodriguez and Jick,[22] Hallas et al.,[23] Silverstein et al.,[24] Hochain et al.,[25] Piper et al.,[26] Shorr et al.,[27] and Barkin.[28]

on univariate analysis and do not consider the interactions among multiple factors and coexisting conditions.

The identification of *Helicobacter pylori* infection as a factor in the development of peptic ulcer has raised the question of a possible synergistic relation between the presence of *H. pylori* infection and NSAID use. Although several studies[29-32] have found these two factors to be independent, two prospective studies have suggested a synergistic relation. Bianchi Porro et al.[33] used the combination of amoxicillin and omeprazole to treat NSAID users infected

with *H. pylori*. They found that the eradication of *H. pylori* did not affect the rate of ulcer healing. However, six months after the end of combination therapy, the cumulative rate of recurrent ulcers was 31 percent among the patients in whom *H. pylori* had been eradicated and 46 percent among those who were still infected. This difference was not statistically significant.

Chan et al.[34] found that the use of a regimen that included bismuth subcitrate to eradicate *H. pylori* significantly decreased the rate of ulcer development associated with the use of naproxen. In this study, gastroduodenal ulcers developed in 26 percent of *H. pylori*–infected persons, but in only 7 percent of those in whom the organism had been eradicated. The inclusion of bismuth in the drug regimen, however, makes the findings somewhat ambiguous, because bismuth can accumulate in the gastric mucosa and stimulate prostaglandin synthesis.[28] Most recently, Hawkey et al.[35] randomly assigned 285 patients with current ulcers or a history of ulcers who were using NSAIDs to combined treatment with omeprazole, clarithromycin, and amoxicillin or to treatment with omeprazole alone. They found that the eradication of *H. pylori* did not affect the rate of recurrent ulcer; in addition, ulcer healing was impaired even in the patients who were successfully treated with antibiotics for *H. pylori* infection. It thus appears that infection with *H. pylori* increases the risk of gastroduodenal mucosal injury associated with NSAID use only minimally, if at all.[28]

Singh et al.[36] recently proposed a simple, point-based algorithm that patients and their physicians can use to estimate the risk of an NSAID-related gastrointestinal complication. If confirmed by other investigators, this tool may help guide decisions about prescriptions for specific NSAIDs, the use of prophylactic agents, and the need for and frequency of patient monitoring.[36]

PATHOGENESIS OF NSAID-INDUCED GASTRODUODENAL MUCOSAL INJURY

Gastroduodenal mucosal injury develops when the deleterious effect of gastric acid overwhelms the normal defensive properties of the mucosa. Concepts about NSAID-induced gastroduodenal mucosal injury have evolved from a simple notion of topical injury to theories involving multiple mechanisms with both local and systemic effects (Fig. 2). The systemic effects are largely the result of the inhibition of endogenous prostaglandin synthesis.[37] Prostaglandin inhibition, in turn, leads to decreases in epithelial mucus, secretion of bicarbonate, mucosal blood flow, epithelial proliferation, and mucosal resistance to injury.[38,39] The impairment in mucosal resistance permits injury by endogenous factors, including acid, pepsin, and bile salts, as well as by exogenous factors such as NSAIDs and possibly ethanol and other noxious agents.

Topical Injury

Mucosal injury is initiated topically by the acidic properties of aspirin and many other NSAIDs. Because of a low dissociation constant, which varies according to the particular agent, these weak acids remain in their nonionized lipophilic form in the highly acidic gastric lumen. Such conditions favor migration through the gastric mucus across plasma membranes and into surface epithelial cells, where NSAIDs are dissociated into the ionized form, resulting in trapping of hydrogen ions.[37] NSAIDs can also cause topical mucosal damage by diminishing the hydrophobicity of gastric mucus, thereby allowing endogenous gastric acid and pepsin to injure the surface epithelium.[39] In addition, topical mucosal injury may occur as a result of indirect mechanisms, mediated through the biliary excretion and subsequent duodenogastric reflux of active NSAID metabolites.[40,41] For example, although sulindac is administered as a nontoxic prodrug, its active metabolite, sulindac sulfide, is excreted into the bile. On entry into the duodenum, sulindac sulfide causes topical injury to the mucosa by virtue of its acidic properties.

The Role of Prostaglandins

Topical injury caused by NSAIDs contributes to the development of gastroduodenal mucosal injury. However, the systemic effects of these agents appear to have the predominant role,[37,42,43] largely through the decreased synthesis of mucosal prostaglandins.[44] The use of enteric-coated aspirin preparations[44] and parenteral[45] or rectal[46] administration of NSAIDs in order to prevent topical mucosal injury has also failed to prevent the development of ulcers. Moreover, doses of aspirin as low as 30 mg are sufficient to suppress prostaglandin synthesis in the gastric mucosa.[47]

Prostaglandins are derived from arachidonic acid, which originates from cell-membrane phospholipids through the action of phospholipase A_2 (Fig. 3). The metabolism of arachidonic acid to prostaglandins and leukotrienes is catalyzed by the cyclooxygenase pathway and the 5-lipoxygenase pathway, respectively.[1,37] Two related but unique isoforms of cyclooxygenase, designated cyclooxygenase-1 and cyclooxygenase-2, have been demonstrated in mammalian cells.[48,49] Despite their structural similarities, they are encoded by distinct genes and differ with regard to their distribution and expression in tissues.[50,51] The cyclooxygenase-1 gene contains a promoter region without a TATA sequence and is primarily expressed constitutively. In contrast, the cyclooxygenase-2 gene is thought to be the inducible form that is nearly undetectable in most (but not all) tissues under normal physiologic conditions.

Cyclooxygenase-1 appears to function as a "housekeeping" enzyme in most tissues, including the gastric mucosa, the kidneys, and the platelets, whereas the expression of cyclooxygenase-2 can be induced

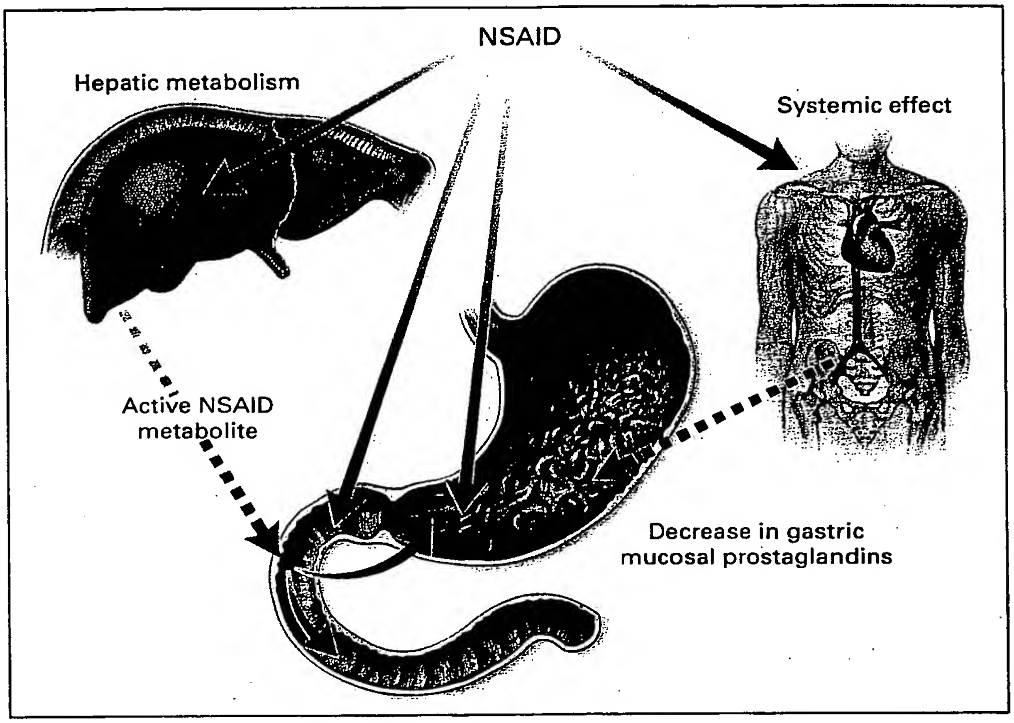

Figure 2. Mechanisms by Which NSAIDs Induce Gastroduodenal Mucosal Injury.
According to the dual-injury hypothesis of Schoen and Vender,[37] NSAIDs have direct toxic effects on the gastroduodenal mucosa (solid arrows) and indirect effects through active hepatic metabolites and decreases in mucosal prostaglandins (broken arrows). Hepatic metabolites are excreted into the bile and subsequently into the duodenum, where they cause mucosal damage to the stomach by duodenogastric reflux and mucosal damage to the small intestine by antegrade passage through the gastrointestinal tract. Adapted from Schoen and Vender.[37]

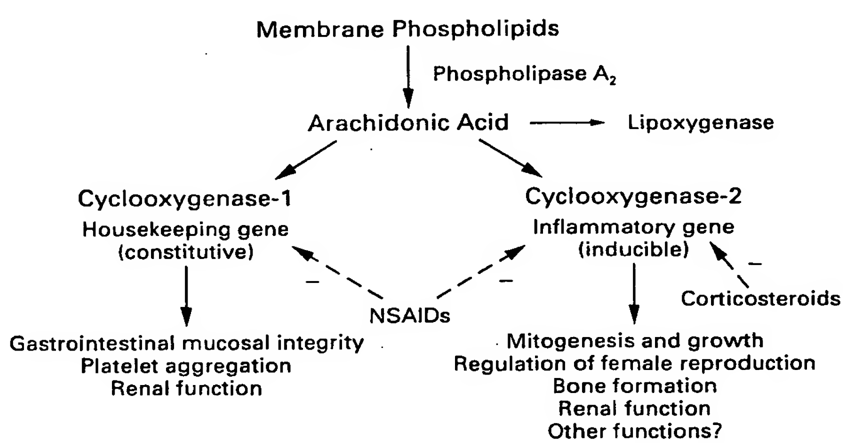

Figure 3. Biosynthesis of Prostaglandins through the Cyclooxygenase Pathways.
The immediate precursor of prostaglandins, arachidonic acid, is derived from membrane phospholipids and is catalyzed by the two cyclooxygenase isoenzymes (also designated as prostaglandin H synthase), cyclooxygenase-1 and cyclooxygenase-2. The gene for cyclooxygenase-1, the housekeeping enzyme, is expressed constitutively and maintains the homeostasis of organs, including gastric mucosal integrity. In contrast, the gene for cyclooxygenase-2, the inflammatory enzyme, is inducible. Although both pathways can be variably inhibited by different NSAIDs, only the gene for cyclooxygenase-2 contains a corticosteroid-responsive repressor element in its promoter region. The broken arrows indicate the inhibitory effects of pharmacologic agents.

by inflammatory stimuli and mitogens in many different types of tissue, including macrophages and synovial cells.[43] It has thus been suggested that the antiinflammatory properties of NSAIDs are mediated through the inhibition of cyclooxygenase-2, whereas adverse effects, such as gastroduodenal ulceration, occur as a result of effects on the constitutively expressed cyclooxygenase-1.[43,49] As discussed below, current strategies for developing NSAIDs with an improved safety profile include the selective inhibition of cyclooxygenase-2, with the sparing of cyclooxygenase-1.

Although there is substantial evidence that the suppression of gastric prostaglandins is the fundamental mechanism responsible for the gastrointestinal toxicity of NSAIDs, some studies suggest that other mechanisms may be involved. For example, ulcers do not develop spontaneously in mice with a disrupted cyclooxygenase-1 gene,[52] and Wallace et al.[53,54] reported that NSAID-induced injury occurred in association with enhanced adherence of neutrophils to the gastric vascular endothelium, as the result of an increase in the expression of intercellular adhesion molecule 1 in the basal endothelium.[55-58] Neutrophil adherence, in turn, causes mucosal injury through the release of oxygen-derived free radicals and proteases.[1]

CLINICAL SPECTRUM OF INJURY

In the majority of patients, NSAID-induced gastroduodenal mucosal injury is superficial and self-limited. However, peptic ulcers develop in some patients, and they may lead to gastroduodenal hemorrhage, perforation, and death. Serious complications of NSAID use that are less commonly recognized include pill esophagitis, small-bowel ulceration, small-bowel strictures, colonic strictures, diverticular disease, and exacerbations of inflammatory bowel disease.[9]

The spectrum of NSAID-related gastroduodenal injury includes a combination of subepithelial hemorrhages, erosions, and ulcerations that is often referred to as NSAID gastropathy. The distinction between erosions and ulcerations depends on pathological definitions, with ulcers defined as lesions that penetrate to the level of the submucosa and erosions defined as lesions confined to the mucosa. For practical purposes, an endoscopic definition is used, which is based on a subjective assessment of the size, shape, and depth of the lesion. Erosions are likely to be small and superficial, whereas ulcers tend to be larger (more than 5 mm in diameter) and deeper.[9]

After ingestion of an NSAID, ultrastructural damage to the gastric surface epithelium occurs within minutes, and gross, endoscopically detectable hemorrhages and erosions in the gastroduodenal epithelium occur within several hours.[59] However, mucosal adaptation appears to occur in response to long-term administration of aspirin in most persons.[60,61] No area of the stomach is resistant to NSAID-induced mucosal injury; the most frequently and severely affected site is the gastric antrum.[59] Although the prevalence and severity of acute injury vary according to the drug formulation,[62-64] the acute injury commonly observed during short-term administration of NSAIDs is not closely correlated with the subsequent development of the more clinically relevant process of mucosal ulceration[20,21,65,66] or with serious complications.[10,67,68] Duodenal mucosal injury occurs less commonly than gastric damage; however, ulcer complications associated with NSAIDs occur with nearly equal frequency in these two sites.[51,66] Prospective, cross-sectional endoscopic studies have shown that the combined prevalence of gastric and duodenal ulcers is 10 to 25 percent in patients with chronic arthritis treated with NSAIDs,[10,67] which is 5 to 15 times the expected prevalence in an age-matched healthy population.

TREATMENT OF NSAID-RELATED DYSPEPSIA

At least 10 to 20 percent of patients have dyspeptic symptoms during NSAID therapy.[10,11] However, such symptoms are poorly correlated with the endoscopic appearance and severity of mucosal injury, since up to 40 percent of persons with endoscopic evidence of erosive gastritis are asymptomatic,[10,68] and conversely, as many as 50 percent of patients with dyspepsia have normal-appearing mucosa.[10]

Histamine H_2–Receptor Antagonists

Several studies using different methods have shown an improvement in dyspeptic symptoms when histamine H_2–receptor antagonists are given to patients taking NSAIDs.[69-73] A recent prospective, observational cohort study by Singh et al.,[11] however, found that asymptomatic patients with rheumatoid arthritis who were taking H_2-receptor antagonists had a significantly higher risk of gastrointestinal complications than those not taking these drugs. The explanation for this surprising observation is unknown, but it might be due to the masking of dyspeptic symptoms associated with mucosal injury. Therefore, although H_2-receptor antagonists are effective in reducing NSAID-related dyspepsia, their routine use in asymptomatic patients taking NSAIDs cannot be recommended. Patients receiving H_2-receptor antagonists for the treatment of dyspepsia must be monitored carefully for the development of serious complications. The initial dose should generally be low (e.g., 400 mg of cimetidine, 150 mg of ranitidine or nizatidine, or 20 mg of famotidine, administered twice daily in each case), and the dose should be tailored to the needs of each patient.

Proton-Pump Inhibitors

In two recent studies, the proton-pump inhibitor omeprazole was compared with ranitidine[74] or mi-

soprostol,[75] a prostaglandin E_1 analogue, for the treatment and prevention of NSAID-related gastroduodenal ulcers. A secondary goal in both of these multicenter trials was to assess the effects of therapy on dyspeptic symptoms. In both studies, although different methods were used to assess the clinical response, omeprazole provided greater symptomatic relief. After four weeks, only 6 percent of patients treated with omeprazole had moderate-to-severe symptoms, as compared with 52 percent at base line, whereas 12 percent of those treated with ranitidine had such symptoms, as compared with 50 percent at base line.[74] A quality-of-life evaluation showed that the patients receiving omeprazole had significantly greater improvement in scores on the Gastrointestinal Symptom Rating Scale than the patients receiving misoprostol.[75] Because proton-pump inhibitors represent a suitable means of preventing the development of gastroduodenal ulcers associated with the use of NSAIDs,[76] they appear to provide a safe and effective form of therapy for NSAID-associated dyspepsia.

MANAGEMENT OF NSAID-RELATED GASTRODUODENAL ULCERS

The optimal treatment for patients with NSAID-induced gastroduodenal ulcers should include the elimination of any potentially aggravating factors. Nontoxic analgesics such as acetaminophen should be substituted for NSAIDs when possible, and in patients with inflammatory arthritides, disease-modifying (or slow-acting) antirheumatic drugs have been recommended as first-line treatment. If NSAID therapy is discontinued, effective treatment aimed at healing the acute ulcer can be instituted with one of several antisecretory agents or with sucralfate. If the use of NSAIDs must be continued, ulcer healing is entirely dependent on the specific agent chosen for ulcer treatment.

Mucosal Protective Agents

Sucralfate, a basic aluminum salt of sucrose octasulfate, is effective in the treatment of both NSAID-related duodenal ulcers and those unrelated to NSAIDs, and the agent appears to be as effective as H_2-receptor antagonists in the healing of non–NSAID-related gastric ulcers.[77] However, sucralfate has no proven benefit in the treatment or prevention of NSAID-related gastric ulcers. Prostaglandins exert their therapeutic effects both by enhancing mucosal defensive properties and by inhibiting gastric-acid secretion.[39] Although they are effective in preventing NSAID-induced gastroduodenal mucosal injury, their role in the treatment of NSAID-associated ulcers is unclear. Hawkey et al.[75] recently compared the capacity of misoprostol (200 μg given four times daily) and omeprazole (20 mg or 40 mg given once daily) to heal gastroduodenal ulcers in patients receiving ongoing NSAID therapy. After eight weeks of therapy, 89 percent of the patients with duodenal ulcers who received omeprazole at either dose had healing, as compared with only 77 percent of those with duodenal ulcers who received misoprostol. Among the patients with gastric ulcers, healing was detected in 80 percent of those receiving 40 mg of omeprazole, in 87 percent of those receiving 20 mg of omeprazole, and in 73 percent of those receiving misoprostol.[75]

Antisecretory Drugs

The efficacy of H_2-receptor antagonists in the treatment of NSAID-related ulcers has not been assessed extensively. Both open, uncontrolled, nonrandomized studies[78] and prospective, randomized studies[79] have suggested that treatment with conventional doses of H_2-receptor antagonists for 6 to 12 weeks results in the healing of approximately 75 percent of gastric ulcers (range, 50 to 88 percent) and 87 percent of duodenal ulcers (range, 67 to 100 percent), despite the continued use of NSAIDs. When the use of NSAIDs is continued, healing appears to be delayed and is largely dependent on the initial size of the ulcer. O'Laughlin et al.[80] reported a 90 percent healing rate for small gastric ulcers (less than 5 mm in diameter) after an eight-week course of treatment with cimetidine, whereas only 25 percent of larger ulcers healed.

In a multicenter trial that included a small subgroup of patients with NSAID-related gastric ulcers, Walan et al.[81] reported that among the patients who continued to receive NSAIDs, the healing rate was higher for those treated with omeprazole than for those treated with ranitidine. A more recent multicenter trial by Yeomans et al.[74] also demonstrated the superiority of omeprazole over ranitidine in the treatment of NSAID-related gastroduodenal ulcers. In this study, the rates of ulcer healing at eight weeks were 79, 80, and 63 percent in the groups receiving 40 mg of omeprazole, 20 mg of omeprazole, and 150 mg of ranitidine twice a day, respectively. A study by Agrawal et al.[82] compared the efficacy of lansoprazole with that of ranitidine in the healing of gastric ulcers during continued NSAID therapy. After eight weeks, ulcers were healed in 57 percent of the patients receiving 150 mg of ranitidine twice daily, whereas ulcers were healed in 73 percent of those receiving 15 mg of lansoprazole once daily and 75 percent of those receiving 30 mg of lansoprazole once daily. These observations suggest that proton-pump inhibitors can heal gastroduodenal ulcers more effectively than H_2-receptor antagonists, whether or not NSAIDs are continued.

PREVENTION OF NSAID-ASSOCIATED GASTRODUODENAL ULCERS

Because of the prevalence and severity of NSAID-related gastrointestinal complications, recent efforts

have been directed at the prevention of mucosal injury induced by NSAIDs. As discussed above, the best way to prevent mucosal injury is to avoid the use of NSAIDs and to substitute an agent less toxic to the gastroduodenal mucosa, such as acetaminophen, salsalate, or magnesium salicylate. Nevertheless, a potent NSAID is commonly preferred, and two strategies have been used to improve their safety: the administration of concomitant medication to protect the gastroduodenal mucosa from injury and the development of safer antiinflammatory agents.

Concomitant Therapy

Sucralfate

Early, small studies suggested that sucralfate might reduce gastroduodenal mucosal injury associated with the use of NSAIDs.[83] However, a large, controlled, randomized trial conducted by Agrawal et al.[84] showed no significant benefit of sucralfate in preventing gastric ulcers in patients with osteoarthritis who were receiving NSAID therapy.

H_2-Receptor Antagonists

Two large, placebo-controlled, prospective trials investigated the protective effect of ranitidine in patients with arthritis who were receiving NSAID therapy.[85,86] Ranitidine (150 mg given twice a day) was effective in preventing duodenal ulcers, which developed in 0 percent and 1.5 percent of the ranitidine-treated patients in the two studies, as compared with 8 percent of the placebo-treated patients in both studies. In contrast, the same dose of ranitidine was ineffective in preventing gastric ulcers in both studies. Taha et al.[73] recently reported a benefit of high-dose famotidine (40 mg given twice a day), as compared with placebo, in preventing both gastric and duodenal ulcers in patients with arthritis who received NSAIDs for 24 weeks. Symptomatic relief was also observed in the group randomly assigned to famotidine, but the benefit, although statistically significant, was only moderate, and the cost of such doses of H_2-receptor antagonists is considerable. Thus, the use of H_2-receptor antagonists for the prevention of NSAID-associated ulcers cannot be recommended.

Proton-Pump Inhibitors

Although proton-pump inhibitors had previously been demonstrated to heal gastroduodenal ulcers effectively in NSAID users,[81] until recently only two small studies[87,88] had systematically examined their effectiveness in preventing NSAID-related gastroduodenal mucosal injury. A recent study compared omeprazole and ranitidine for the prevention of recurrent gastroduodenal ulcers in a large number of patients with arthritis in whom NSAID therapy could not be discontinued.[74] After six months of treatment, 16.3 percent of the patients treated with ranitidine had gastric ulcers, and 4.2 percent had duodenal ulcers. In the omeprazole group, only 5.2 percent of the patients had gastric ulcers, and only 0.5 percent had duodenal ulcers.[74]

Another recent study compared omeprazole (20 mg given once a day) and misoprostol (200 μg given twice a day) for the prevention of recurrent ulcers in patients with arthritis who were receiving NSAID therapy.[75] After six months, 12 percent of the patients receiving placebo and 10 percent of those receiving misoprostol, but only 3 percent of those receiving omeprazole, had duodenal ulcers. Gastric ulcers recurred in 32 percent of the patients receiving placebo, in 10 percent of those receiving misoprostol, and in 13 percent of those receiving omeprazole.[75] These studies suggest that, like misoprostol, proton-pump inhibitors are superior to H_2-receptor antagonists. Although a prospective analysis of clinical outcomes has not been performed, these agents appear to be effective in preventing the recurrence of ulcers during continued use of NSAIDs.[76]

Prostaglandins

In their initial study, Graham et al.[67] reported that the prevalence of gastric ulcers in patients with osteoarthritis who were receiving NSAIDs was 1.4 percent in those receiving concomitant treatment with 200 μg of misoprostol four times a day, 5.6 percent in those receiving 100 μg of misoprostol four times a day, and 21.7 percent in those receiving placebo. The efficacy of misoprostol as prophylaxis against duodenal ulcers was confirmed in a subsequent study by Graham et al.[89] Despite the effectiveness of misoprostol in preventing gastroduodenal ulcers, the agent was not associated with any improvement in dyspeptic symptoms in these studies. Furthermore, diarrhea developed in many of the patients receiving the 200-μg dose of misoprostol. Raskin et al.[90] compared three regimens of misoprostol (200 μg given twice, three times, or four times a day) and concluded that although lower doses of misoprostol are better tolerated, the drug needs to be taken at least three times a day to provide effective prophylaxis against NSAID-induced gastric ulcers.

It must be emphasized that the prevention of endoscopically detectable ulcers as an end point is not necessarily a safeguard against the development of serious ulcer-related complications. To determine whether treatment with misoprostol could affect the incidence of ulcer complications caused by NSAID use, Silverstein et al.[24] conducted the Misoprostol Ulcer Complication Outcomes Safety Assessment (MUCOSA) study. They reported a 40 percent reduction in the overall rate of complications due to NSAID-associated ulcers in a group of patients receiving 200 μg of misoprostol four times a day, as compared with the patients receiving placebo.[24]

Although misoprostol is highly effective for preventing NSAID-induced ulcers and is the only drug

approved by the Food and Drug Administration as prophylaxis against NSAID-related gastroduodenal ulcers, it has a number of adverse effects. These include diarrhea and abdominal pain associated with the increased generation of cyclic adenosine monophosphate in the small intestine and increased uterine contractility that can lead to spontaneous abortion.

Development of Safer NSAIDs

Several modifications in the formulation of NSAIDs have been introduced in recent years to reduce their toxicity. Recent surveillance and endoscopic studies have confirmed that the incidence of gastroduodenal mucosal injury is reduced with the use of nabumetone, etodolac, and meloxicam.[91-93] The improved safety of meloxicam appears to be due to its preferential inhibition of cyclooxygenase-2, with a minimal effect on cyclooxygenase-1. In contrast, nabumetone and etodolac appear to inhibit cyclooxygenase-2 preferentially at low doses, but the preferential inhibition of cyclooxygenase-2 is diminished at higher doses. These agents also have other properties that contribute to their safety. Etodolac has a low level of enterohepatic recirculation and a short half-life; nabumetone is a nonacidic prodrug formulation and has no enterohepatic recirculation.[94]

Highly Selective Cyclooxygenase-2 Inhibitors

Highly selective cyclooxygenase-2 inhibitors have recently been developed that, in studies to date, have had a markedly reduced capacity to cause injury to the gastroduodenal mucosa.[95-98] Two of the compounds, celecoxib and rofecoxib, have been studied extensively, and they appear to maintain their selectivity for cyclooxygenase-2 at doses substantially higher than those required to affect inflammation. These agents are more than 100 times as selective in their ability to inhibit cyclooxygenase-2 as the currently available NSAIDs and have been shown to promote the development of gastroduodenal ulcers at a rate not significantly different from that of placebo.[99,100] The selectivity ratios for inhibition of cyclooxygenase-1 and cyclooxygenase-2 of celecoxib, rofecoxib, and other agents have been determined primarily by in vitro assays.[101] Although these drugs have similar in vivo selectivity, genetic differences among patients may affect the cyclooxygenase-2 selectivity of these drugs. Celecoxib became available for use in the United States in February 1999, and rofecoxib will probably be available very soon.

In spite of enthusiasm for these promising new NSAIDs, some questions remain regarding their highly selective inhibition of cyclooxygenase-2. For example, cyclooxygenase-2 might generate endogenous prostanoids that are biologically important (Fig. 3). Mice in which the gene for cyclooxygenase-2 has been disrupted have defects in renal function and regulation of bone resorption, and female mice have impaired reproductive physiology.[94] Mizuno et al.[102] have suggested that an increase in mucosal cyclooxygenase-2 expression may be necessary for the normal healing of gastroduodenal ulcers. However, nonselective NSAIDs also inhibit cyclooxygenase-2 to varying degrees, and the critical factor may be the ratio of isoenzyme inhibition.

McAdam et al.[103] recently reported that celecoxib, but not ibuprofen, suppressed the urinary excretion of prostacyclin in healthy subjects, whereas thromboxane activity related to cyclooxygenase-1 was suppressed only by ibuprofen. The authors speculated that long-term therapy with these agents might increase the rate of thrombotic events in patients who were at increased risk for cardiovascular disease, although no data were collected on such events.[103] On a positive note, the expression of cyclooxygenase-2 messenger RNA is enhanced in human colorectal adenomas and adenocarcinomas, and selective cyclooxygenase-2 inhibition may thereby reduce the risk of colorectal cancer.[104] The results of these studies indicate that although the highly selective cyclooxygenase-2 inhibitors offer considerable promise in the treatment of inflammatory arthritides, careful surveillance will be important to determine their ultimate benefit and safety profile.

NSAIDs Containing Nitric Oxide

Nitric oxide has a critical role in maintaining the integrity of the gastroduodenal mucosa, exerting many of the same effects as endogenous prostaglandins.[105-107] It has even been suggested that nitric oxide and prostaglandins may act synergistically to mediate mucosal protective effects,[1] and Salvemini et al.[108] have demonstrated that nitric oxide stimulates cyclooxygenase enzymes. Such redundancy in preserving normal physiologic function is not unique, and it constitutes the rationale for the development of formulations in which nitric oxide is released and compensates for the suppression of mucosal prostaglandins. Under these conditions, the desired effects of NSAIDs are maintained, including the inhibition of both cyclooxygenase isoenzymes, while toxicity is minimized (Fig. 4).[109-111] Nitric oxide–containing compounds have antiinflammatory and antipyretic activities that are similar to those of the parent compound and may have analgesic effects that are greater than those of the parent compound.[110]

In a recent seven-day clinical trial, a flurbiprofen–nitric oxide formulation was found to cause fewer gastric erosions than the parent drug, with the same inhibitory effects on gastric mucosal prostaglandin synthesis and serum thromboxane levels.[112] In addition, nitric oxide, like aspirin, inhibits platelet aggregation, but it does not suppress cyclooxygenase activity or cause gastric mucosal injury.[113] The use of nitric oxide–aspirin compounds as prophylaxis against

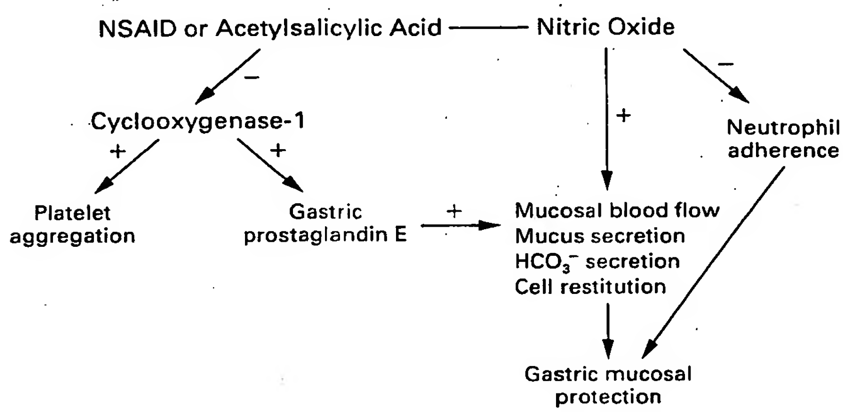

Figure 4. Postulated Mechanism by Which Nitric Oxide–Releasing NSAIDs Maintain the Ability to Protect the Gastroduodenal Mucosa while Suppressing the Level of Endogenous Mucosal Prostaglandins.
Nitric oxide appears to stimulate some of the defensive properties of the mucosa that are affected by inhibition of the cyclooxygenase-1 isoenzyme. In addition, nitric oxide inhibits intercellular adhesion molecule 1, thereby decreasing neutrophil adherence, resulting in the prevention of NSAID-induced gastroduodenal mucosal injury. Adapted from Wallace.[1]

Table 2. Current Recommendations for the Treatment of NSAID-Related Dyspepsia and Mucosal Injury.

Clinical Situation	Recommendation
Dyspepsia	Empirical treatment with H_2-receptor antagonist (e.g., 400 mg of cimetidine, 150 mg of ranitidine or nizatidine, or 20 mg of famotidine, all twice daily) or proton-pump inhibitor (e.g., 20 mg of omeprazole, 30 mg of lansoprazole, 20 mg of rabeprazole, or 40 mg of pantoprazole daily before breakfast); individualize therapy
Helicobacter pylori infection	Treatment to eradicate infection only in patients with a history of peptic ulcer
Active gastroduodenal ulcer NSAID discontinued	Treatment with an H_2-receptor antagonist (e.g., 800 mg of cimetidine, 150 mg of ranitidine or nizatidine, or 40 mg of famotidine daily before bedtime) or a proton-pump inhibitor (as above)
NSAID continued	Treatment with a proton-pump inhibitor (as above)
Prophylactic therapy	Concomitant treatment with misoprostol (≥200 μg three times a day), a proton-pump inhibitor (as above), or a cyclooxygenase-2–preferential or cyclooxygenase-2–selective NSAID

myocardial and cerebrovascular ischemia is also under investigation.

Other Approaches

Several other compounds are being developed, including NSAIDs associated with zwitterionic phospholipids, chiral NSAIDs, basic fibroblast growth factor, and trefoil peptides.[94] Although initial studies indicate that some of these compounds may help reduce the gastrointestinal toxicity of NSAIDs, their clinical use awaits further investigation.

SUMMARY

Recommendations for the prevention and management of gastroduodenal mucosal injury associated with NSAIDs are proposed in Table 2. Symptoms associated with the use of NSAIDs are common and can generally be treated empirically with an H_2-receptor antagonist or a proton-pump inhibitor. Although additional studies are necessary, eradication of *H. pylori* should be reserved for patients with a history of ulcer disease. In general, if a gastroduodenal ulcer develops, the most prudent approach is to discontinue

the NSAID and substitute therapy with acetaminophen or a nonacetylated salicylate. If treatment with the NSAID must be continued, proton-pump inhibitors should be used, since they appear to heal ulcers at the same rate, whether or not NSAID therapy is continued. After the ulcer has healed and it has been determined that NSAID therapy must be continued, the most effective prophylaxis against recurrent ulcers is the concomitant administration of misoprostol (at least 200 μg given three times a day) or a proton-pump inhibitor, or the use of an NSAID that preferentially or selectively inhibits cyclooxygenase-2. The ultimate choice of therapy in a particular patient depends on several things, including risk factors, the preferences of the patient and the physician, and cost. The development of cyclooxygenase-2–selective inhibitors and the formulation of other new, safer NSAIDs should broaden the range of options.

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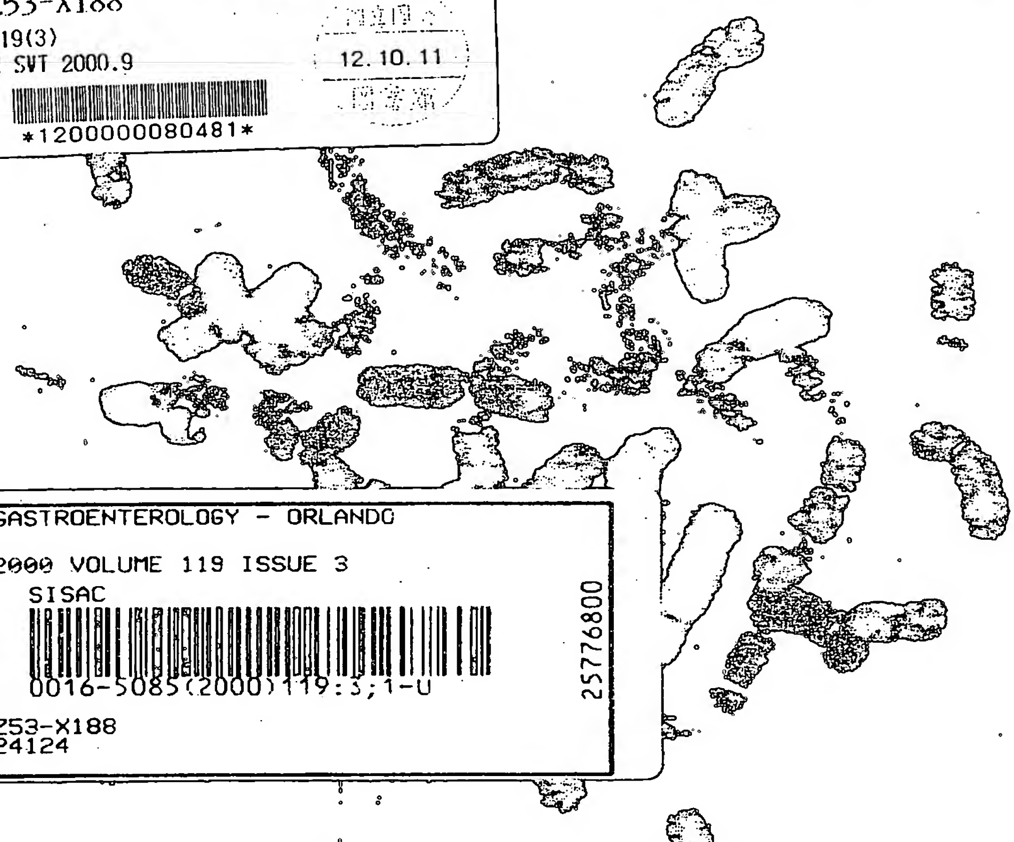

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NSAID-Induced Gastric Damage in Rats: Requirement for Inhibition of Both Cyclooxygenase 1 and 2

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Background & Aims: Selective cyclooxygenase (COX)-2 inhibitors produce less gastric damage than conventional nonsteroidal anti-inflammatory drugs (NSAIDs), suggesting that NSAIDs cause damage by inhibiting COX-1. We tested this hypothesis in rats by using a selective COX-1 inhibitor (SC-560). **Methods:** The effects of SC-560, celecoxib (selective COX-2 inhibitor), or a combination of both inhibitors on gastric damage and prostaglandin synthesis were determined. Selectivity of the drugs for COX-1 vs. COX-2 was assessed in the carrageenan-airpouch model. A COX-1–preferential inhibitor, ketorolac, was also evaluated. The effects of these inhibitors on leukocyte adherence to vascular endothelium and on gastric blood flow were assessed. **Results:** SC-560 markedly reduced gastric prostaglandin synthesis and platelet COX-1 activity, but spared COX-2 and did not cause gastric damage. Celecoxib did not affect gastric prostaglandin E_2 synthesis and did not cause gastric damage. However, the combination of SC-560 and celecoxib invariably caused hemorrhagic erosion formation, comparable to that seen with indomethacin. Ketorolac caused damage only at doses that inhibited both COX isoforms, or when given with a COX-2 inhibitor. Celecoxib, but not SC-560, significantly increased leukocyte adherence, whereas SC-560, but not celecoxib, reduced gastric blood flow. **Conclusions:** Inhibition of both COX-1 and COX-2 is required for NSAID-induced gastric injury in the rat.

The identification of two isoforms of cyclooxygenase (COX) has led to a reevaluation of the mechanism through which nonsteroidal anti-inflammatory drugs (NSAIDs) cause injury to the gastric mucosa. The observation that selective inhibition of COX-2 spares gastric prostaglandin (PG) synthesis and is associated with a greatly reduced incidence of gastric erosions compared with what is observed with conventional NSAIDs[1,2] has led to the hypothesis that it is the suppression of gastric COX-1 by NSAIDs that is the key mechanism responsible for erosion formation.[3–5] However, this remains an unproven hypothesis. Mice in which the gene for COX-1 is disrupted exhibit greatly reduced gastric PG synthesis, but no gastric injury.[6] Although it is possible that the lack of gastric damage in these mice is attributable to compensatory changes in mucosal defense in response to the reduced PG synthesis, one cannot rule out the possibility that reduced COX-1 activity, alone, is not sufficient for erosion formation. Indeed, when given indomethacin, a dual inhibitor of COX-1 and COX-2, these mice did develop gastric erosions.[6]

Many studies in recent years have suggested that COX-2 can contribute to gastric mucosal defense, at least in some circumstances. For example, Gretzer et al.[7] reported that selective COX-2 inhibitors interfered with adaptive response of the gastric mucosa to a topical irritant. Normally, the topical irritant increased the resistance of the gastric mucosa to damage induced by subsequent administration of a damaging agent, such as absolute ethanol. However, when pretreated with a selective COX-2 inhibitor, this protective response was inhibited, despite the fact that significant inhibition of gastric PG synthesis could not be detected.[7] COX-2 is expressed in the human stomach colonized by *Helicobacter pylori*, and it has been suggested that the PGs derived from COX-2 play a role in protecting the stomach against damage associated with this infection.[8] A role of COX-2–derived PGs in gastric ulcer healing is supported by studies in experimental models.[9–11] Because all conventional NSAIDs inhibit both COX-1 and COX-2 when administered at doses effective in reducing inflammation and pain,[5] it is possible that inhibition of both of these isozymes in the gastric mucosa contributes to the generation of erosions and ulcers.

Recently, Smith et al.[12] described the effects of a selective inhibitor of COX-1 (SC-560) in rat models of pain and inflammation. This compound was found to markedly reduce gastric PG content, but surprisingly, the effects on mucosal integrity were not mentioned. In the present study, we used this selective inhibitor of

Abbreviations used in this paper: COX, cyclooxygenase; ELISA, enzyme-linked immunosorbent assay; TX, thromboxane.

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COX-1 and selective inhibitors of COX-2 (celecoxib and DuP-697) to test the following hypothesis: suppression of both COX-1 and COX-2 is necessary for NSAID-induced gastric damage in the rat stomach. We have also performed studies with ketorolac, the most COX-1–selective of the currently marked NSAIDs,[5] to determine the relative contributions of inhibition of COX-1 vs. COX-2 to the generation of gastric damage. Finally, because both leukocyte adherence and reduced gastric blood flow have been suggested to contribute to the pathogenesis of NSAID-induced gastric damage,[13] the effects of the selective COX inhibitors on leukocyte adherence to the vascular endothelium in vivo and on gastric blood flow were examined.

Materials and Methods

Animals

Male Wistar rats weighing 175–200 g were obtained from Charles River Breeding Farms (Montreal, Quebec, Canada) and were housed in the Animal Care Facility at the University of Calgary. The rats were fed standard laboratory chow and tap water ad libitum. The rats were deprived of food, but not water, for 18–20 hours before an experiment. All experiments described below received prior approval from the Animal Care Committee of the University of Calgary and were performed in accordance with the guidelines of the Canadian Council on Animal Care.

Selection of Doses of Test Drugs

A series of experiments was performed to establish the doses of SC-560 and celecoxib that would produce significant and selective inhibition of COX-1 and COX-2, respectively. The carrageenan-airpouch model has previously been used in our laboratory to determine the extent of suppression of COX-2 in vivo.[14] The PGE_2 that can be recovered in the inflammatory exudate is derived almost entirely from COX-2.[14] We have previously found that doses of celecoxib in the 5–45 mg/kg range significantly reduced the levels of PGE_2 in the inflammatory exudate without affecting whole blood thromboxane (TX) synthesis (an index of COX-1 activity).[15] In the present study, we used the airpouch model to examine the effects of celecoxib at a dose of 15 mg/kg, SC-560 at doses of 10–40 mg/kg, and the combination of these two drugs (same doses; n = 5–8 per group). For comparison, we also tested the effects of indomethacin (5 mg/kg), a dual inhibitor of COX-1 and COX-2. In some studies, a second selective inhibitor of COX-2, DuP-697, was used. We have previously shown that the dose used (10 mg/kg) suppressed COX-2 activity in vivo by more than 80% while having no effect on COX-1 activity.[14] Control rats were treated with vehicle (1% carboxy methylcellulose). One hour after oral administration of the test drugs or vehicle, carrageenan (2 mL of a 1% solution) was injected into the airpouch. Six hours later, the rats were anesthetized with halothane and the airpouch was carefully opened by an incision. The exudate fluid was collected for measurement of PGE_2 concentration by enzyme-linked immunosorbent assay (ELISA).[16] A 1-mL sample of blood was incubated at 37°C for 45 minutes then centrifuged (1000*g*; 10 minutes). The concentration of TXB_2 in the supernatant was measured by ELISA.[16]

Acute Gastric Damage

Groups of at least 5 rats each were given one of the following orally: celecoxib (15 mg/kg), SC-560 (20 or 40 mg/kg), the combination of these two drugs (same doses), or indomethacin (5 mg/kg). Control rats received an equal volume of the vehicle (1% carboxymethylcellulose). Three hours later, the rats were anesthetized with halothane and a blood sample was drawn from the inferior vena cava for measurement of whole blood TX synthesis, as described above. The stomach was removed and scored for hemorrhagic damage, by an observer unaware of the treatments the rats had received. The scoring involved measuring the lengths of all lesions, in millimeters, and summing the values to give an overall gastric damage score for each rat. A sample of the corpus region of the stomach was then excised, weighed, and added to a tube containing 1 mL of sodium phosphate buffer (10 mmol/L; pH 7.4). The tissue sample was minced with scissors for 30 seconds, then placed in a shaking water bath (37°C) for 20 minutes. The samples were centrifuged (9000*g*) for 1 minute, and the concentration of PGE_2 in the supernatant was determined by ELISA.[16]

Effects of Ketorolac

Of the NSAIDs presently on the market, ketorolac, shows the greatest selectivity for COX-1.[5] To further examine the importance of inhibition of COX-1 and COX-2 in the pathogenesis of NSAID-induced gastric injury, we examined the effects of a range of doses of ketorolac on gastric PGE_2 synthesis, whole blood TXB_2 synthesis (as an index of COX-1), inflammatory PGE_2 synthesis in the carrageenan-airpouch model (as an index of COX-2), and gastric damage. The experiments were performed in the same manner as described above. Ketorolac was tested at doses of 0.3, 1, 3, 10, and 30 mg/kg. Each group consisted of 5–6 rats.

To complement the studies of acute gastric damage induced by the combination of SC-560 and celecoxib, similar experiments were performed in which rats were given ketorolac at a dose that selectively inhibited COX-1 (3 mg/kg) alone or in combination with a selective COX-2 inhibitor (celecoxib at 15 mg/kg or DuP-697 at 10 mg/kg). As above, the rats were killed 3 hours later for blind scoring of the gastric damage.

The damage induced by acute administration of NSAIDs consists of erosions in the corpus region of the stomach. In contrast, chronic gastric ulcers induced by NSAIDs in humans are found primarily in the antrum. To determine if suppression of COX-1 and COX-2 is necessary for antral ulcer formation, we used the model of NSAID-induced antral ulceration originally described by Satoh et al.[17] Groups of rats (n = 5) were fasted for 20 hours, then given access to food for 2 hours. At

the end of the period of feeding, the rats were given the test drugs orally and were then fasted for another 24 hours. The rats were killed, and the stomach was examined by an observer unaware of the treatments they had received. The presence of antral ulceration and hemorrhage was noted. The test drugs used in this study were ketorolac (3 mg/kg; a dose selective for COX-1), celecoxib (15 mg/kg), the combination of ketorolac (3 mg/kg) and celecoxib (15 mg/kg), or ketorolac at a dose that suppressed both COX-1 and COX-2 (10 mg/kg).

Intravital Microscopy

The effects of celecoxib and SC-560 on leukocyte adherence to the vascular endothelium were examined using an intravital microscopy preparation, as described previously.[18] Rats (n = 5–6 per group) were anesthetized with sodium pentobarbital (65 mg/kg intraperitoneally [IP]). Images of the mesenteric microcirculation were recorded for 5 minutes after a 15-minute equilibration period. The mesentery was then superfused with bicarbonate-buffered saline containing celecoxib at 1 or 3 μmol/L, SC-560 at 0.3 or 1 μmol/L, the combination of SC-560 at 1 μmol/L and celecoxib at 3 μmol/L, indomethacin at 7 μmol/L, or buffer alone. The images were recorded for 5 minutes beginning 15, 30, 45, and 60 minutes after the start of the superfusion. The 3 μmol/L concentration of celecoxib was selected because it has been reported to be in the range of plasma concentrations required for anti-inflammatory effects in humans[19] and is considerably less than the concentration necessary for significant anti-inflammatory effects in the carrageenan-induced paw edema model.[12] SC-560 has been reported to inhibit COX-1 at a concentration of 0.3 μmol/L[12] and has been shown to inhibit intestinal PG synthesis in the rat at this concentration (Dr. W. MacNaughton, personal communication, April 2000). Leukocyte adherence was quantified on video playback in a blind manner. A leukocyte was considered adherent to the endothelium if it remained stationary for 30 seconds or more. Vessel diameter was monitored throughout the experiment using a digital caliper.[18]

To confirm the effectiveness of SC-560 in inhibiting COX-1 at the concentrations tested, and to rule out the possibility that celecoxib inhibited COX-1 in a similar setting to these experiments, the following experiment was performed. Blood was drawn from the inferior vena cava of 4 rats and distributed to tubes containing (final concentration) celecoxib (3 μmol/L), SC-560 (1 μmol/L), indomethacin (7 μmol/L), or vehicle. The samples were incubated for 45 minutes at 37°C, then centrifuged (1000*g*, 10 minutes). TXB_2 concentrations in the supernatant were measured by ELISA.

Gastric Blood Flow

Conventional NSAIDs have been shown to cause a decrease in gastric blood flow[20–22]; this has been suggested to contribute significantly to the pathogenesis of injury associated with these agents.[13] To determine if selective inhibition of COX-1 or COX-2 would result in a decrease in gastric blood flow, experiments were carried out in which gastric blood flow was measured by laser-Doppler flowmetry, as described in detail previously.[23] An ex vivo gastric chamber preparation was used.[23] The exposed stomach was bathed with 100 mmol/L hydrochloric acid throughout the experiment. A laser-Doppler probe was placed on the surface of the dorsal, corpus region of the stomach for continuous recording of blood flow.[23] After a 15-minute basal period, SC-560 (40 mg/kg), celecoxib (15 mg/kg), the combination of SC-560 and celecoxib, indomethacin (5 mg/kg), or vehicle was injected IP (n = 4–6 rats per group). Blood flow over the hour that followed was expressed as a percentage of the flow rate in the basal period.

Statistical Analysis

All data are expressed as mean ± SEM. Comparisons among groups of data were performed using a one-way analysis of variance followed by the Dunnett's Multiple Comparison test. An associated probability (*P* value) of <5% was considered significant.

Materials

SC-560 was provided by Dr. F. Degner of Boehringer-Ingelheim (Ingelheim, Germany). Celecoxib was obtained from Monsanto (St. Louis, MO), and ketorolac tromethamine was obtained from Roche Laboratories (Montreal, Quebec, Canada). The ELISA kits for PGE_2 and TXB_2 were obtained from Cayman Chemical Co. (Ann Arbor, MI). λ-Carrageenan was obtained from Sigma Chemical Co. (St. Louis, MO). All other reagents were obtained from Fisher Scientific (Edmonton, Alberta, Canada).

Results

Selectivity of the Inhibitors

In the carrageenan-airpouch model, celecoxib inhibited PGE_2 synthesis by 97% but had no effect on whole blood TX synthesis (Figure 1). Thus, at this dose, celecoxib acted as a selective COX-2 inhibitor. In contrast, SC-560 caused almost complete inhibition (>95% inhibition) of whole blood TX synthesis at all doses tested (10–40 mg/kg), confirming its ability to inhibit COX-1. At the 40 mg/kg dose, SC-560 suppressed TX synthesis by 99% (Figure 1). However, SC-560 failed to significantly affect inflammatory PGE_2 synthesis, which has been shown to be almost exclusively derived from COX-2.[14] Indomethacin significantly inhibited COX-1 and COX-2, because both whole blood TX and inflammatory PGE_2 synthesis were markedly suppressed (Figure 1).

Gastric PG Synthesis

Celecoxib had no effect on PGE_2 synthesis in the normal stomach at doses in the 1–4 mg/kg range (Figure 2 shows data for the 15 mg/kg dose). SC-560 at a dose of 20 mg/kg did not significantly affect gastric PGE_2 syn-

thesis (14.2 ± 2.8 vs. 23.4 ± 3.5 pg/mg in vehicle-treated group). However, at a dose of 40 mg/kg, SC-560 significantly inhibited gastric PGE_2 synthesis by 70% (Figure 2). The combination of celecoxib (15 mg/kg) and SC-560 (40 mg/kg) caused a similar degree of inhibition of gastric PGE_2 synthesis as was seen with SC-560 alone (67%; Figure 2). Indomethacin inhibited gastric PGE_2 synthesis by 65% (Figure 2).

Because we could not detect any effect of celecoxib on gastric PG synthesis in healthy rats, possibly because any contribution of COX-2 to PG synthesis was negligible with respect to the contribution of COX-1, we examined the effects of celecoxib in a setting of increased gastric COX-2 expression. Gastric ulcers were induced by serosal application of acetic acid, as described in detail previously.[24] On the seventh day after induction of the ulcer, groups of 6 rats each received either celecoxib (15 mg/kg) or vehicle. Three hours later, the rats were killed and the stomach was removed. A sample of the gastric tissue from the ulcer margin was excised, and PGE_2 synthesis was measured as described in Materials and Methods. In the vehicle-treated rats, an average of 223 ± 36 pg/mg tissue of PGE_2 was released by the tissue sample. In celecoxib-treated rats, this was reduced by 48% (to 116 ± 23 pg/mg; $P < 0.05$).

Gastric Damage

In previous studies we observed that celecoxib at doses of 5, 15, or 45 mg/kg selectively inhibited COX-2 in vivo and did not produce any detectable gastric damage in the rat.[14] Based on those studies, we selected the 15 mg/kg dose for the present study and confirmed that it did not produce macroscopically (Figure 2) or histologically detectable gastric damage. Similarly, SC-560 at doses of 10, 20, or 40 mg/kg did not elicit damage in the stomach. Histological evaluation of gastric tissue fixed 3 hours after administration of SC-560 (40 mg/kg) confirmed the absence of any damage. When examined in a blind manner, the tissues from rats treated with SC-560 were indistinguishable from the tissues from rats treated with vehicle. In contrast, the combination of SC-560 (40 mg/kg) and celecoxib resulted in the development of gastric erosions in all 10 rats, with a mean gastric damage score that was significantly greater than seen in the other groups (Figure 2). The combination of celecoxib with the 20 mg/kg dose of SC-560 failed to cause

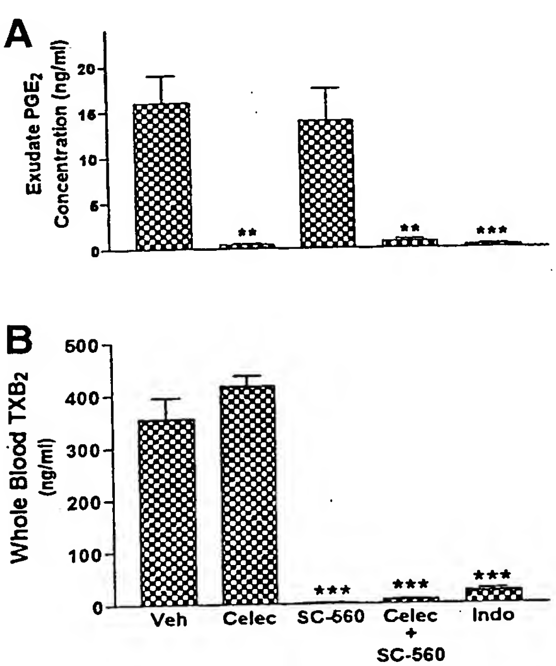

Figure 1. Effects of celecoxib (15 mg/kg) and SC-560 (40 mg/kg) on inflammatory PGE_2 synthesis (index of COX-2 activity; *A*) and whole blood TX synthesis (index of COX-1 activity; *B*). Celecoxib inhibited COX-2 but not COX-1. SC-560 inhibited COX-1 but not COX-2. The combination of celecoxib and SC-560 inhibited both COX-1 and COX-2. Indomethacin (5 mg/kg) inhibited COX-1 and COX-2 to similar extents as the two selective inhibitors. $^{**}P < 0.01$, $^{***}P < 0.001$ vs. vehicle-treated group. n = 5–8/group.

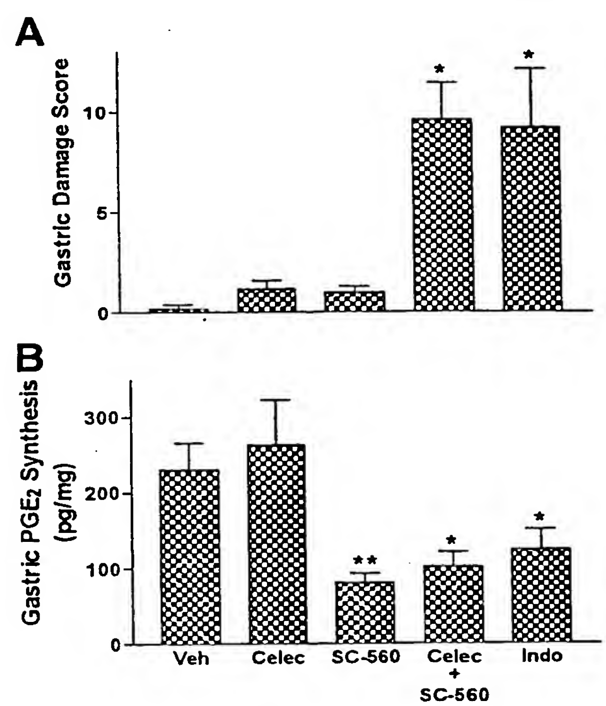

Figure 2. Effects of celecoxib (selective COX-2 inhibitor; 15 mg/kg) and SC-560 (selective COX-1 inhibitor; 40 mg/kg) on gastric mucosal integrity (gastric damage; *A*) and gastric PGE_2 synthesis (*B*). Significant increases in gastric damage were observed only in the group treated with celecoxib plus SC-560 and in the group treated with indomethacin (5 mg/kg). $^{*}P < 0.05$, $^{**}P < 0.01$ vs. vehicle-treated group. n = 5–10/group.

significant gastric damage (mean score, 0.2 ± 0.2). Indomethacin also caused gastric damage, the extent of which was similar to that observed with the combination of SC-560 and celecoxib (Figure 2).

Because it was possible that the increased level of damage observed in rats given both celecoxib and SC-560 was caused by topical irritant properties of celecoxib, we performed an additional experiment in which rats (n = 5 per group) were given SC-560 orally at 40 mg/kg, and celecoxib (15 mg/kg) or vehicle IP. Damage was scored 3 hours later, as in the studies described above. In the rats receiving SC-560 orally and vehicle IP, the mean damage score was 0.2 ± 0.2. In contrast, the rats that received oral SC-560 and IP celecoxib had a mean gastric damage score of 7.4 ± 1.6 ($P < 0.05$).

Effects of Ketorolac and DuP-697

As shown in Figure 3, all doses of ketorolac tested produced significant inhibition of COX-1 activity (TX synthesis) and gastric PG synthesis. Doses of ≥1 mg/kg inhibited COX-1 activity by 95% and gastric PG synthesis by >88%. Despite this, significant gastric damage was not observed with ketorolac at doses in the 0.3–3 mg/kg range. Ketorolac did not significantly affect COX-2 activity (inflammatory PG synthesis in the air-pouch model) at doses of ≤3 mg/kg. With doses of 10 and 30 mg/kg, ketorolac produced significant inhibition of COX-2 activity (by 75% and 91%, respectively) and produced significant gastric damage.

Having identified a dose of ketorolac that inhibited COX-1 but not COX-2, we then tested the effects of the combination of that dose of ketorolac with a selective COX-2 inhibitor. As shown in Figure 4, administration of ketorolac together with celecoxib resulted in significant gastric damage. Moreover, administration of ketorolac with DuP-697, another selective COX-2 inhibitor,[14] also resulted in significant gastric damage. DuP-697 alone failed to cause gastric damage.

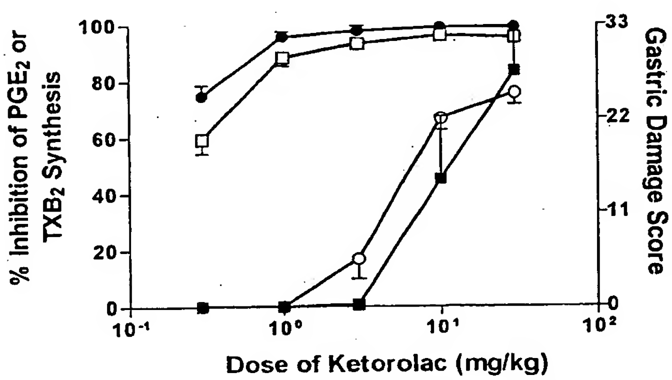

Figure 3. Inhibition of eicosanoid synthesis and gastric damaging effects of ketorolac. Effects on COX-1 activity (●; whole blood TX synthesis), COX-2 activity (○; inflammatory PGE_2 synthesis in the carrageenan-airpouch model), gastric PGE_2 synthesis (□), and gastric damage with various doses of ketorolac (■; 0.3–30 mg/kg) were assessed. Gastric PGE_2 synthesis and COX-1 activity were significantly ($P < 0.01$) suppressed at all doses of ketorolac. COX-2 activity was significantly suppressed ($P < 0.05$) only at the 10 and 30 mg/kg doses of ketorolac, the same doses that caused significant gastric damage (compared with a vehicle-treated group). Each group consisted of 6 rats.

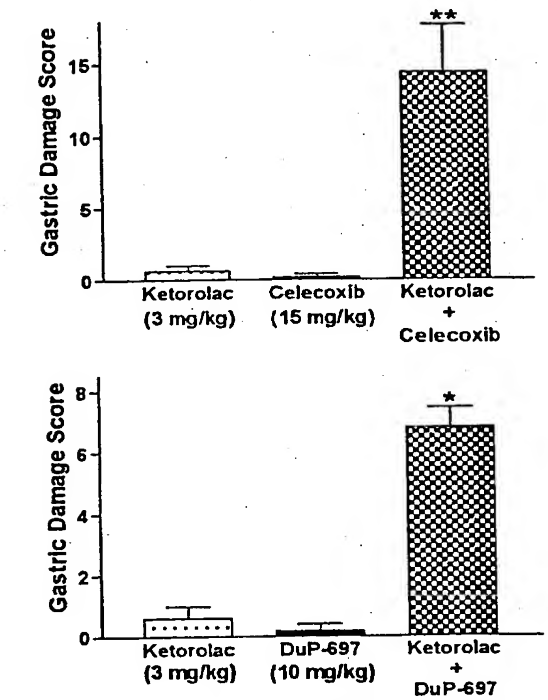

Figure 4. Gastric damaging effects of ketorolac, at a dose selectively inhibiting COX-1, DuP-697 (a selective COX-2 inhibitor), celecoxib (a selective COX-2 inhibitor), and the combination of ketorolac and one of the two COX-2 inhibitors. $*P < 0.05$, $**P < 0.01$ compared with groups treated with only one of the test drugs. n = 5–6/group.

We also evaluated the effects of selective inhibitors of COX-1 and COX-2 in a model in which ulceration of the antrum of the stomach is produced. Using this "refeeding" model,[17] rats given celecoxib alone (15 mg/kg) or ketorolac alone (3 mg/kg) did not exhibit any detectable damage. However, 4 of 5 rats given the combination of these two drugs developed antral damage and overt hemorrhage. Moreover, all 5 rats given a higher dose of ketorolac (10 mg/kg), which inhibits both COX-1 and COX-2 (Figure 3), developed hemorrhagic antral ulceration.

Effects of COX Inhibitors on Leukocyte Adherence

Celecoxib (3 μmol/L) caused a 6-fold increase in leukocyte adherence over basal levels (Figure 5). A significant increase in leukocyte adherence was evident within 30 minutes of beginning the superfusion of the

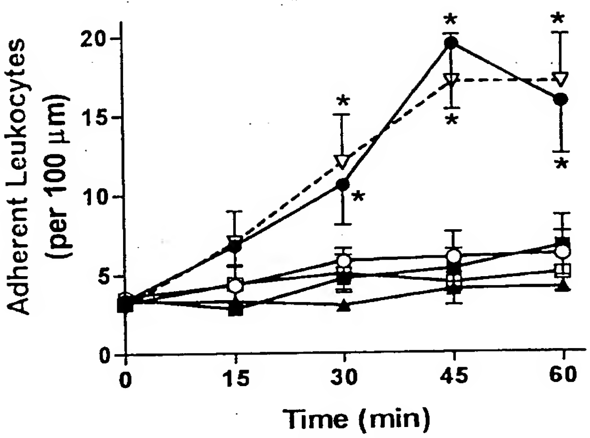

Figure 5. Effects of 1 μmol/L (○) and 3 μmol/L (●) celecoxib (selective COX-2 inhibitor), 0.3 μmol/L (□) and 1 μmol/L (■) SC-560 (selective COX-1 inhibitor), and 7 μmol/L (▽) indomethacin (dual COX-1 and COX-2 inhibitor) on leukocyte adherence to the vascular endothelium of mesenteric venules in the rat. After a basal period, the vessels were superfused with one of these inhibitors, with vehicle (▲), or with indomethacin, for a period of 60 minutes. Indomethacin (7 μmol/L) and celecoxib (3 μmol/L) significantly increased the numbers of adherent leukocytes. A combination of SC-560 and celecoxib resulted in increases in leukocyte adherence similar to that seen with SC-560 alone. $*P < 0.05$ vs. vehicle-treated group. n = 5–6/group.

blood vessels. The lower concentration of celecoxib (1 μmol/L) did not produce a statistically significant change in leukocyte adherence. SC-560 did not cause an increase in leukocyte adherence at either concentration tested (0.3 or 1 μmol/L). Indomethacin (7 μmol/L) caused adherence similar in magnitude to that seen with the higher concentration of celecoxib. Superfusion with the combination of SC-560 (1 μmol/L) and celecoxib (3 μmol/L) caused leukocyte adherence similar in magnitude to that seen with celecoxib alone.

None of the test drugs significantly affected the diameter of the mesenteric venules during the 60-minute exposure period. The mean diameter at the beginning of the experiment in all groups ranged between 30 and 35 μm. At the end of the 60-minute exposure to one of the test drugs of the vehicle, the mean diameters in the various groups ranged, as a percentage of the starting diameter, from 97% to 105% (no significant differences among the groups and no significant change from the respective basal value). Confirmation that the 3 μmol/L concentration of celecoxib did not inhibit COX-1 activity was provided by the experiments in which whole blood was exposed to this concentration of celecoxib for 45 minutes, and the generation of TXB_2 was then measured. TXB_2 synthesis in this group averaged 7.04 ± 0.10 ng · mL^{-1} · min^{-1}, compared with 5.24 ± 0.11 ng · mL^{-1} · min^{-1} in whole blood to which only the vehicle was added. On the other hand, SC-560 at a concentration of 1 μmol/L inhibited TXB_2 synthesis by 98% (0.09 ± 0.02 ng · mL^{-1} · min^{-1}). Exposure of whole blood to indomethacin (7 μmol/L) also resulted in profound inhibition of TXB_2 synthesis (0.13 ± 0.04 ng · mL^{-1} · min^{-1}; 98% inhibition).

Effects on Gastric Blood Flow

IP administration of vehicle did not cause any significant changes in gastric blood flow over the following 60-minute period (Figure 6). Similarly, administration of celecoxib (15 mg/kg IP) did not produce any significant changes in gastric blood flow. The mean rate of gastric blood flow 60 minutes after celecoxib administration was 101% ± 12% of the basal flow rate. In contrast, administration of SC-560 (40 mg/kg IP) or indomethacin (5 mg/kg) resulted in a profound reduction in gastric blood flow. In both cases, blood flow had decreased to ~50% of basal levels by 1 hour after administration ($P < 0.01$). Coadministration of SC-560 (40 mg/kg) and celecoxib (15 mg/kg) resulted in a decrease in gastric blood flow comparable with that seen with SC-560 alone. By 1 hour after administration of the drugs, gastric blood flow had decreased to 52% ± 4% of basal levels.

Discussion

Selective inhibition of COX-2 has been shown to be associated with significantly less gastric erosion formation than that seen with anti-inflammatory doses of

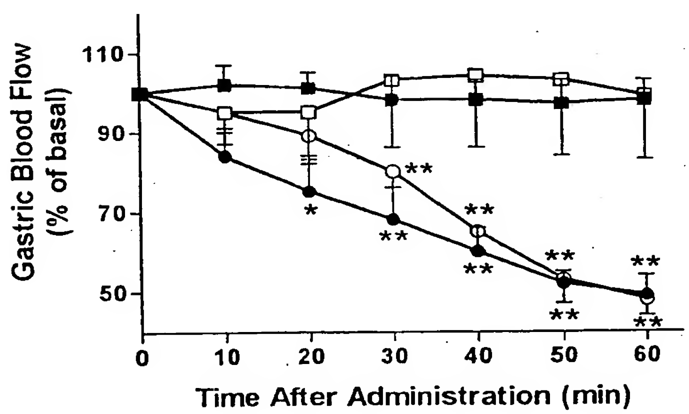

Figure 6. Effects of celecoxib (□; selective COX-2 inhibitor; 15 mg/kg IP), SC-560 (●; selective COX-1 inhibitor; 40 mg/kg IP), or indomethacin (○; 5 mg/kg IP) on gastric blood flow in the rat. After a basal period, one of the inhibitors or vehicle (■) was injected and blood flow was continuously measured by laser-Doppler flowmetry for 60 minutes. The combination of SC-560 and celecoxib resulted in decreases in gastric blood flow similar to that seen with SC-560 alone. The gastric blood flow data are expressed as a percent of the basal flow in each rat. $*P < 0.05$, $**P < 0.01$ vs. vehicle-treated group. n = 4–6/group.

conventional NSAIDs, both in animals[1,2] and humans.[4,25] Such data are consistent with the notion that the suppression of COX-1 by conventional NSAIDs underlies their ability to cause gastric damage. However, it is a presumption that blockade of COX-1 is all that is needed for erosions to develop, because before the present study, the effects of a selective COX-1 inhibitor on gastric mucosal integrity have not been reported. Even excluding the potential contribution of topical irritant properties of NSAIDs, it remained possible that the ability of these agents to inhibit COX-2 activity may also be an important component of the mechanism of action in terms of inducing gastric damage. The results of the present study suggest that inhibition of both COX-1 and COX-2 is required for the development of gastric erosions after NSAID administration in the rat. Neither a COX-1 inhibitor nor a COX-2 inhibitor caused macroscopically or histologically detectable gastric damage when given at doses that were proven to be effective at selectively inhibiting the target enzyme in vivo. However, administration of both inhibitors invariably resulted in gastric erosion development. Moreover, a COX-1–preferential inhibitor, ketorolac, was shown to cause gastric damage only when given doses in which significant inhibition of COX-2 occurred. These doses were 10–30-fold greater than the doses required for near-complete inhibition of COX-1 and gastric PG synthesis. Ketorolac (at a dose that selectively blocked COX-1) given together with DuP-697 (another selective COX-2 inhibitor) produced significant gastric damage, whereas neither drug alone caused damage. Likewise, the combination of ketorolac and celecoxib produced significant gastric damage (in the corpus and antrum), whereas neither drug alone caused significant damage.

The results of the present study also show that there are distinct mechanisms through which inhibition of COX-1 vs. COX-2 could contribute to erosion formation. We have previously proposed that NSAID-induced adherence of neutrophils to the vascular endothelium within the gastric microcirculation contributes to the generation of mucosal injury.[18,26,27] The induction of neutrophil adherence by NSAIDs is likely to be in part caused by suppression of the tonic production of PGs (such as prostacyclin) by the vascular endothelium.[28] Given the numerous reports that COX-1 is the constitutively expressed isoform of COX, responsible for "housekeeping functions,"[1–3] it was our presumption that the ability of NSAIDs to stimulate neutrophil adherence was caused by suppression of COX-1 in the vascular endothelium. The results of the present study suggest that this is not the case. Indeed, the selective COX-2 inhibitor, celecoxib, elicited significant leukocyte adherence in mesenteric venules that was comparable with that achieved with a conventional NSAID (indomethacin), whereas the selective COX-1 inhibitor (SC-560) did not. In the latter case, >98% suppression of COX-1 was confirmed through measurement of whole blood TX synthesis. Whether the effect of celecoxib is caused by suppression of COX-2 in the endothelium or in another cell type is not clear. Interestingly, however, the possibility that COX-2 is a major source of prostacyclin synthesis is supported by a recent human study in which >80% of prostacyclin synthesis in healthy volunteers could by inhibited by celecoxib.[19] Significant inhibition of prostacyclin synthesis by therapeutic doses of rofecoxib has also been shown.[29]

A decrease in gastric blood flow after NSAID administration has been documented in laboratory animals and humans,[20–22,30] and has been suggested to contribute significantly to the pathogenesis of mucosal injury.[13] The demonstration that SC-560, but not celecoxib, produced a decrease in gastric blood flow in the rat strongly suggests that the effect of NSAIDs is caused by suppression of COX-1. Both the time course and magnitude of the decrease in gastric blood flow observed with SC-560 were similar to what occurs with indomethacin and what we have previously observed with diclofenac.[30]

The conclusion that suppression of both COX-1 and COX-2 is necessary for NSAID-induced gastric damage in the rat is consistent with a number of previous findings. For example, mice in which the gene for COX-1 was disrupted, so that they did not have functional COX-1, did not exhibit spontaneous gastric damage despite negligible gastric PG synthesis. However, these mice did develop erosions when given indomethacin (a dual COX-1/COX-2 inhibitor). Warner et al.[5] recently evaluated more than 40 NSAIDs, comparing their effects on the two COX isoforms using a human whole blood assay. While they pointed to the suppression of COX-1 as a key to gastric toxicity of NSAIDs, which we do not dispute, they also showed that when tested in whole blood at concentrations that suppressed COX-2 activity by 80%, all of the conventional NSAIDs also markedly suppressed COX-1 activity (i.e., by more than 60%). Thus, despite the fact that several drugs (including ketorolac) exhibited considerable selectivity for COX-1 in vitro, these drugs would still act as nonselective inhibitors when used at doses that are effective for reducing pain and inflammation. The only other study we are aware of in which a selective COX-1 inhibitor was evaluated was that of Smith et al.[12] They showed that SC-560 could almost completely abolish gastric PG con-

tent, but conspicuous by its absence was any mention of the effects of this drug on gastric mucosal integrity. In that study, doses of SC-560 as high as 100 mg/kg were used.

As previously suggested by others,[7] our results are consistent with the hypothesis that PGs derived from COX-2 contribute to mucosal defense. However, we were not able to detect any greater reduction of gastric PGE_2 synthesis when both a COX-1 and COX-2 inhibitor were given vs. the effect of the COX-1 inhibitor alone. This is consistent with the findings of Gretzer et al.,[7] who observed an effect of several COX-2 inhibitors on mucosal resistance to injury, but could not detect any significant change in gastric PG synthesis. The most likely explanation for these findings is that the contribution of COX-2 to total PG synthesis in the normal stomach is very small (not detectable using the assay system we used, which is a measure of PG synthetic capacity), but is nevertheless very important in terms of mucosal defense. This explanation is supported by the demonstrated ability of celecoxib to inhibit PG synthesis in the stomach in a model (acetic acid–induced gastric ulcer) in which COX-2 expression is markedly up-regulated.[8]

In this study we focused on the suppression of COX activity by NSAIDs as a mechanism of gastric injury. Of course, NSAIDs have other actions unrelated to suppression of COX that contribute to damage. For example, many NSAIDs exert topical irritant effects that can contribute to mucosal injury.[31] Indeed, it was possible that topical irritant properties of celecoxib, together with the suppression of gastric PG synthesis by SC-560, accounted for the damage we observed when both drugs were given. However, IP administration of celecoxib together with oral administration of SC-560 still produced mucosal damage, suggesting that the inhibition of COX-2 by celecoxib, rather than topical irritant properties, accounted for the damage induced when the two compounds were given together. Similarly, the combination of orally administered ketorolac (at dose specific for COX-1) and IP DuP-697 (at dose specific for COX-2) caused significantly more gastric damage than either drug alone. As was the case with oral administration, the extent of damage observed with the combination of a COX-1 inhibitor and a COX-2 inhibitor was increased in a synergistic, rather than additive, manner.

In summary, this study shows that selective inhibition of either COX-1 or COX-2 does not elicit gastric damage in the rat; rather, inhibition of both isoforms of COX is required for NSAID-induced damage to develop. The results obtained with SC-560 and celecoxib were confirmed with other inhibitors of COX-1 (ketorolac) and COX-2 (DuP-697). Both COX isoforms seem to contribute to mucosal defense. We have identified distinct mechanisms through which inhibition of these isoforms may contribute to the pathogenesis of NSAID-induced gastric damage. COX-1 inhibition results in reduced gastric blood flow, whereas COX-2 inhibition leads to increased leukocyte adherence to the vascular endothelium. These results suggest that the concept that inhibition of COX-1 alone is the mechanism underlying NSAID-induced gastric injury is incorrect. Although the notion that anti-inflammatory drugs that are COX-1 sparing[32] will be less toxic to the stomach may be accurate, this term may be misconstrued such that an important contribution of COX-2 to gastric mucosal defense is overlooked.

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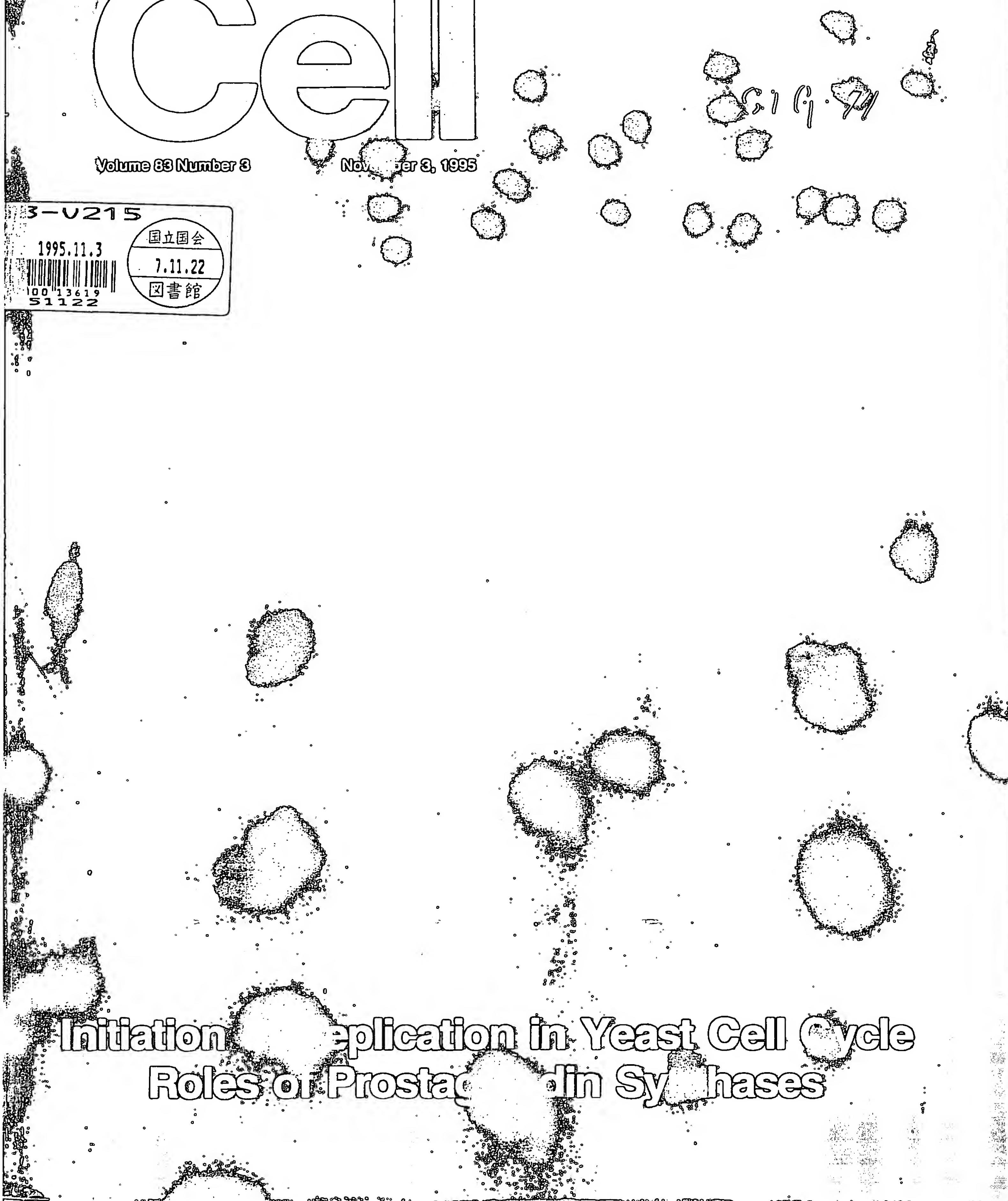

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Prostaglandin Synthase 1 Gene Disruption in Mice Reduces Arachidonic Acid-Induced Inflammation and Indomethacin-Induced Gastric Ulceration

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Summary

Cyclooxygenases 1 and 2 (COX-1 and COX-2) are key enzymes in prostaglandin biosynthesis and the target enzymes for the widely used nonsteroidal anti-inflammatory drugs. To study the physiological roles of the individual isoforms, we have disrupted the mouse *Ptgs1* gene encoding COX-1. Homozygous *Ptgs1* mutant mice survive well, have no gastric pathology, and show less indomethacin-induced gastric ulceration than wild-type mice, even though their gastric prostaglandin E_2 levels are about 1% of wild type. The homozygous mutant mice have reduced platelet aggregation and a decreased inflammatory response to arachidonic acid, but not to tetradecanoyl phorbol acetate. *Ptgs1* homozygous mutant females mated to homozygous mutant males produce few live offspring. COX-1-deficient mice provide a useful model to distinguish the physiological roles of COX-1 and COX-2.

Introduction

The cyclooxygenase isoforms, COX-1 and COX-2, catalyze the key step in the synthesis of prostaglandins. While COX-1 and COX-2 catalyze the same reaction, the conversion of arachidonic acid (AA) to prostaglandin H_2 (PGH_2), the isoforms appear to have different biological roles. COX-1 and COX-2 are also the target enzymes for the most widely used drugs in human medicine, nonsteroidal anti-inflammatory drugs (NSAIDs). Thus, there is considerable interest in understanding the physiological roles of COX-1 and COX-2 and in developing drugs that differentially inhibit them.

The COX isoforms are encoded by genes located on different chromosomes (Wen et al., 1993). The gene encoding COX-1 (*Ptgs1* in the mouse) spans about 22 kb (Yokoyama and Tanabe, 1989; Kraemer et al., 1992), from which a message of 2.8 kb is derived (DeWitt and Smith, 1988; Merlie et al., 1988; Yokoyama et al., 1988). The gene encoding COX-2 (*Ptgs2* in the mouse) is about 8 kb (Xie et al., 1991; Kujubu et al., 1991; O'Banion et al., 1991) and produces a message of about 4.1 kb (Xie et al., 1991; Kujubu et al., 1991; O'Banion et al., 1991; Kujubu and Herschman, 1992; DuBois et al., 1994b). The COX-1 and COX-2 proteins from the same species are about 60% identical with the catalytic regions being conserved.

The genes encoding COX-1 and COX-2 differ in their regulation at the transcriptional level, and recent data reinforce the likelihood that the isoforms mediate different biological functions (Morita et al., 1995; Murakami et al., 1994). COX-1 appears to be constitutively synthesized in many tissues (Simmons et al., 1991; O'Neill and Ford-Hutchinson, 1993; Seibert et al., 1994), although its level of expression can vary with the state of differentiation or following cytokine or tumor promoter stimulation (Smith et al., 1993, 1994; Samet et al., 1995; Murakami et al., 1995). COX-1 is thought to carry out primarily "housekeeping" functions such as cytoprotection of the gastric mucosa, regulation of renal blood flow, and platelet aggregation (DeWitt and Smith, 1988, 1990; Merlie et al., 1988; Funk et al., 1991). In contrast, COX-2 message and protein are normally undetectable in most tissues; however, COX-2 expression in certain cell types can be rapidly induced by proinflammatory or mitogenic agents, including cytokines, endotoxins, tumor promoters, and mitogens (Xie et al., 1991; O'Banion et al., 1992; Hla and Neilson, 1992; Fletcher et al., 1992; DuBois et al., 1994a; Smith et al., 1994). Because of this rapid induction, the gene encoding COX-2 has been termed an immediate-early response or primary response gene (Simmons et al., 1989; Maier et al., 1990; Fletcher et al., 1992; Ryseck et al., 1992). COX-2 message and protein have been shown to be up-regulated in human colon cancers (Eberhart et al., 1994; Kargman et al., 1995) and in mouse skin papillomas and carcinomas (Muller-Decker et al., 1995).

The COX isoforms are the primary target enzymes for NSAIDs, which act by inhibiting the activity of the enzymes (Vane, 1971; Smith and Willis, 1971; Smith et al., 1990; Xie et al., 1992; Seibert et al., 1994; Masferrer et al., 1994; Mitchell et al., 1994; Seibert and Masferrer, 1994). Aspirin, the most common and best-studied NSAID, was originally shown to inhibit prostaglandin synthesis by Vane (1971). NSAIDs in common use today include aspirin, ibuprofen, and indomethacin, and all inhibit the COX enzymes. In addition to the use of NSAIDs as analgesics and for alleviation of acute and chronic inflammatory diseases such as arthritis (Levi and Shaw Smith, 1994; Simon, 1994), NSAIDs (in particular, aspirin) have proven effective for decreasing the frequency of heart attacks and strokes (Vane and Botting, 1992) and in reducing the incidence of colon cancer (Thun et al., 1991, 1993; Marnett, 1992). Some NSAIDs also inhibit chemically induced colon cancer in rodents (Rao et al., 1995, and references therein).

From the combined evidence of NSAID effects on rodent and human colon cancers, it has been suggested that randomized prevention trials with humans be initiated (Heath et al., 1994). However, while NSAIDs have many beneficial effects, they can also cause adverse side effects, the most common of which are gastrointestinal ulceration and nephrotoxicity (Clive and Stoff, 1984; Black, 1986; Brooks and Day, 1991; Price and Fletcher, 1990; Simon, 1994).

Since the discovery of COX-2, the identification of drugs that selectively inhibit this isoform has become the focus of NSAID development (Xie et al., 1992; Meade et al., 1993; Seibert and Masferrer, 1994; Mitchell et al., 1994; Masferrer et al., 1994). The rationale for this is that COX-1 is not elevated during inflammation and that, as a necessary housekeeping gene, its inhibition by NSAIDs may be the cause of their adverse side effects. In contrast, COX-2 is normally nondetectable in most tissues, but is rapidly elevated during inflammation (Masferrer et al., 1994; Crofford et al., 1994; Vane et al., 1994; Mitchell et al., 1994; Seibert et al., 1994; Vane, 1994; Simmons et al., 1991), and its inhibition by NSAIDs is thought responsible for their therapeutic effects.

The relative biological contributions of the COX-1 and COX-2 isoforms in the maintenance of normal physiological functions and in diseased states is not, however, entirely clear. Most of the current knowledge has come from studies of NSAID inhibition, COX-2 induction, or both. As an alternative approach to understand the roles of these enzymes better, we have used gene targeting to generate a mouse strain that is unable to synthesize COX-1. The development of COX-2-deficient mice is reported by Morham et al. (1995 [this issue of *Cell*]). With these mice, we hope to learn more about the roles of the two isoforms in normal physiology and in various inflammatory disorders and to understand better the etiology of the therapeutic effects and deleterious side effects of NSAIDs. In the present study, we report the generation of mice lacking COX-1 and some of their phenotypic characteristics.

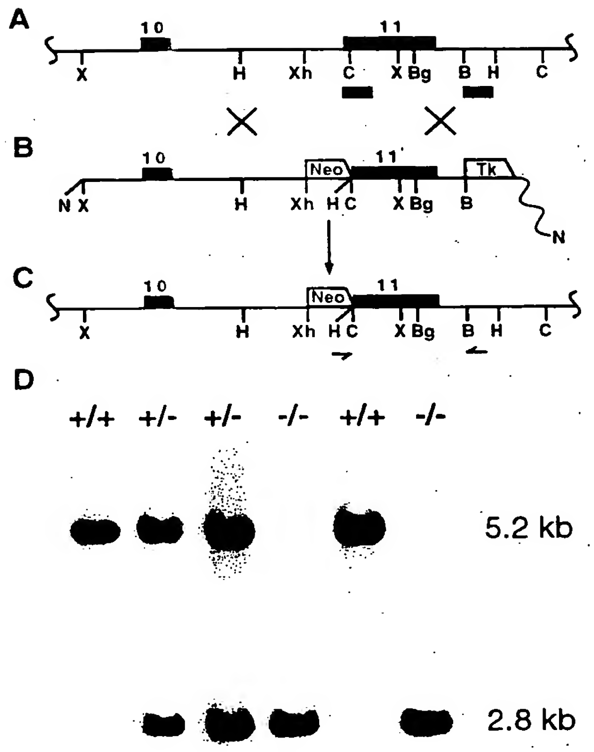

Figure 1. Targeted Disruption of the Mouse *Ptgs1* Gene

(A) The 3′ region of the *Ptgs1* gene. Closed boxes represent exons, and bars below represent the probes. Restriction sites: Bg, BglII; B, BamHI; C, ClaI; H, HindIII; X, XbaI; Xh, XhoI.

(B) Targeting construct. Phosphoglycerolkinase-promoted neomycin and herpes simplex thymidine kinase genes are represented by Neo and Tk, respectively. Wavy lines represent plasmid (not to scale). N shows the linearizing NotI restriction site.

(C) Targeted *Ptgs1* locus. Diagnostic PCR primers are indicated by arrows.

(D) Southern blot of HindIII-digested targeted allele (2.8 kb) and wild-type allele (5.2 kb) mouse tail DNA. Wild type (+/+), heterozygotes (+/−), and homozygote mutants (−/−) are shown.

Results

Cloning of the 3′ Region of the *Ptgs1* Gene

The 3′ region of the mouse *Ptgs1* gene was cloned using a 357 bp probe synthesized from strain 129 mouse embryonic stem (ES) cell DNA. The probe was made by polymerase chain reaction (PCR) with primers for the 5′ end of exon 11, the sequences of which were based on the human *COX-1* gene structure (Yokoyama and Tanabe, 1989) and the mouse cDNA sequence (DeWitt et al., 1990). This probe detected only a single band on Southern blots of mouse ES cell DNA digested with XbaI, HindIII, BglII, BamHI, or SacI, indicating that it was specific for a single gene. The 15 kb fragment cloned was subsequently shown to be from *Ptgs1*, and not the isoform *Ptgs2*, by the following. First, three different primer pairs specific for sequences in exon 11 of *Ptgs1* were used for PCR, and the predicted product sizes were obtained. Second, about 100 bp of DNA 3′ of the ClaI site in exon 11 (Figure 1A) was sequenced from the fragment and shown to match the published sequence of *Ptgs1* (DeWitt and Smith, 1990). Third, PCR with primers specific for the 5′ and 3′ ends of *Ptgs1* exon 10 produced the correct sized product from the cloned fragment. Fourth, the 2.4 kb PCR fragment used to diagnose targeted clones (Figure 1C) was digested by BglII into fragments of the predicted sizes.

Vector Construction and Targeting

The targeting strategy for disruption of the *Ptgs1* gene is shown in Figure 1. Since aspirin inactivates COX-1 by acetylating Ser-530 (Humes et al., 1981; Roth et al., 1983; DeWitt et al., 1990), we disrupted the gene prior to the codon for this amino acid in exon 11. The targeting vector was designed to replace about 1 kb of intron 10, together with the splice junction and first 44 bp of exon 11, with the neomycin resistance (*Neo*) gene (Figure 1B). If a pro-

tein were made from the resulting disrupted gene, it would lack the carboxy-terminal 120 amino acids, including Ser-530. Alternate splicing to eliminate the *Neo* gene would result in the elimination of 14 amino acids and loss of proper reading frame.

The plasmid was linearized with NotI and electroporated into strain 129-derived E14TG2a ES cells. Following electroporation, G418 and ganciclovir selection was started (Mansour et al., 1988), and 6 of 96 doubly resistant colonies isolated were positive for the expected 2.4 kb PCR product indicated in Figure 1C. Using the exon 11 probe indicated in Figure 1A, we confirmed targeting in these PCR-positive clones by detection on Southern blots of the expected 2.8 kb HindIII fragment. The same 2.8 kb band was also detected with a 510 bp BamHI–HindIII fragment that hybridizes to a genomic region 3′ to the targeting construct (Figure 1A).

Production of Animals

Cells from two of the targeted clones were injected into C57BL/6J (B6) blastocysts, resulting in the birth of four male chimeras. One chimera produced heterozygous F1 129/B6 offspring after mating to B6 females. From the first seven F2 litters obtained by mating F1 heterozygous siblings, 14 wild-type, 31 heterozygous, and 16 homozygous mutant weanling pups were obtained, in agreement with Mendelian expectations ($p > 0.9$). Southern blots of HindIII-digested tail DNA from wild-type F2, heterozygous F2, and homozygous mutant F2 mice are shown in Figure 1D.

General Health of the COX-1-Deficient Mice

The COX-1-deficient mice develop normally and appear healthy. Necropsy and microscopic examination of selected tissues (liver, spleen, kidney, gastrointestinal tract, reproductive tract, heart, and lungs) from three homozygous mutant males and three homozygous mutant females, aged 2–5 months, revealed no significant pathology. However, in three of six kidneys examined from homozygous mutant mice, a minimal change was present, characterized by one or two small foci per section of basophilic, immature tubules. The size and frequency of these lesions did not change with age. All six wild-type age-matched controls did not show these foci. There were no consistent or remarkable findings in other tissues examined, including the glandular and nonglandular stomach.

Northern Blot Analysis of the COX-1 Message

The effect of *Ptgs1* gene disruption on *COX-1* mRNA was analyzed by determining the level of message in the colons and kidneys of wild-type, heterozygous, and homozygous mutant F2 mice using the 357 bp probe described above. Figure 2A shows that the level of full-length (2.8 kb) message is reduced to approximately half normal in the heterozygous mice. No 2.8 kb message is detected in the homozygous mutant mice. Similar results (data not shown) were obtained when the 1.7 kb fragment of mouse cDNA (Oxford Biomedical) was used as the probe. When the blots were rehybridized to a probe specific for the *Neo* gene,

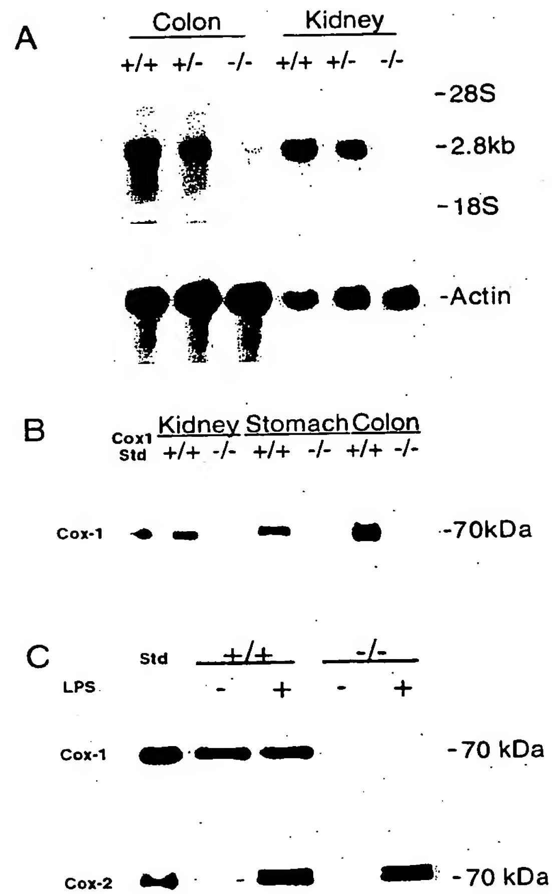

Figure 2. Northern and Western Blot Analyses

(A) Northern blot of total RNA from colon and kidney of wild-type (+/+), heterozygous (+/−), and homozygous mutant (−/−) mice. The blot was probed with a 357 bp PCR fragment from the *COX-1* cDNA.
(B) Western blot of microsomal protein from kidney, stomach, and colon of wild-type (+/+) and homozygous mutant (−/−) mice. The COX-1 standard is in the left lane. The protein was detected with a polyclonal antibody to COX-1.
(C) Western blot of total protein from control and LPS-stimulated peritoneal macrophages from wild-type (+/+) and homozygous mutant (−/−) mice. (Top) COX-1 levels in control and LPS-stimulated peritoneal macrophages from wild-type and homozygous mutant mice; a COX-1 standard is on the left; the protein was detected with an antibody to COX-1. (Bottom) COX-2 levels in control and LPS-stimulated peritoneal macrophages from wild-type and homozygous mutant mice; a COX-2 standard is on the left; the protein was detected with a polyclonal antibody to COX-2.

a band at 1.1 kb was detected (data not shown) in the heterozygous and homozygous mutant RNA samples; as expected, the band was more intense in the homozygous mutant sample. The *Neo* probe did not hybridize with any band detected by the *COX-1* probes.

Western Blot Analysis of the COX-1 Protein

A polyclonal antibody against residues 274–289 of COX-1 (Morita et al., 1995) was used to determine the level of

COX-1 protein or any truncated product from the disrupted gene that includes these residues. Amino acids 274–289 would still be present in a truncated protein if it were made, as the codons for these amino acids are 5′ to the site of gene disruption. Western blot analysis (Figure 2B) shows that the normal 70 kDa COX-1 protein is readily detectable in kidney, stomach, and colon microsomes of wild-type F2 *Ptgs1* mice. Neither normal-sized COX-1 protein nor any smaller fragment is detected in the same tissues from the homozygous mutant mice. Figure 2C (upper panel) demonstrates that COX-1 protein levels are not significantly affected by lipopolysaccharide (LPS) in macrophages from wild-type mice and, as expected, are not detectable in the homozygous mutant mice. The bottom panel of Figure 2C shows that LPS induces the COX-2 protein about equally in peritoneal macrophages from COX-1 wild-type and homozygous mutant mice. COX-2 message and PGE_2 production are also induced about equally in the macrophages from wild-type and homozygous mutant mice (data not shown). These results indicate that disruption of *Ptgs1* prevents constitutive synthesis of COX-1 but does not alter *Ptgs2* inducibility in macrophages.

PGE_2 Production in Peritoneal Macrophages

To determine the effect of *Ptgs1* gene disruption on prostaglandin biosynthesis, we isolated and analyzed peritoneal macrophages for their basal (not LPS-stimulated) level of PGE_2 production with exogenous AA as the substrate. The data in Figure 3 show that basal PGE_2 production is reduced about 70% in heterozygous mice and is reduced more than 99% in homozygous mutant mice. The basal levels of PGE_2 production in the wild-type and heterozygous mice were not altered by a 6 hr incubation in medium containing dexamethasone, indicating that PGE_2 production is due to COX-1 and not to COX-2, the production of which is inhibited by dexamethasone. These data show that basal PGE_2 production is virtually absent in macrophages from the *Ptgs1* disrupted mice and that COX-2 contributes little to basal levels of PGE_2 from exogenous AA in unstimulated macrophages.

Gastric Ulceration

The COX-1 homozygous mutant mice from F2 and subsequent generations did not have gross or microscopic gastric lesions; therefore, the absence of COX-1 alone is not sufficient to cause lesions. Because COX-1 is a target for NSAIDs (Xie et al., 1992; Seibert et al., 1994; Masferrer et al., 1994; Mitchell et al., 1994; Seibert and Masferrer, 1994) and because NSAID inhibition of COX-1 has been thought causal in the induction of gastrointestinal lesions, we investigated the possibility that COX-1-deficient mice have altered sensitivity to indomethacin, an NSAID known to induce stomach ulceration in mice (Yokoyama et al., 1985; Rainsford, 1987; Ettarh and Carr, 1993). First, a dose response was determined by administering indomethacin to heterozygous F2 mice by gavage at doses ranging from 10 to 80 mg/kg (data not shown). Doses of 10 and 20 mg/kg were found to be in the lower, but still detectable, response range. The data in Figure 4 show that F2 and F3 wild-type and homozygous mutant mice treated with 20 mg/kg have about an equal number of ulcers, although the percent of surface area ulcerated is somewhat reduced in the homozygous mutant mice (p <

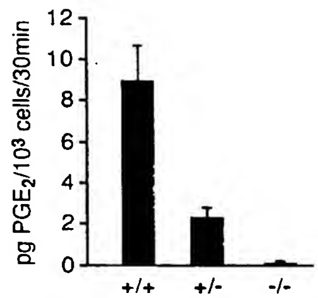

Figure 3. Production of PGE_2 by Peritoneal Macrophages

PGE_2 levels were determined by radioimmunoassay, and the data are expressed as picograms of PGE_2 per 10^3 cells. Data are for macrophages from five mice for each genotype (wild type, heterozygous, and homozygous mutant). Data are presented as the mean ± SEM.

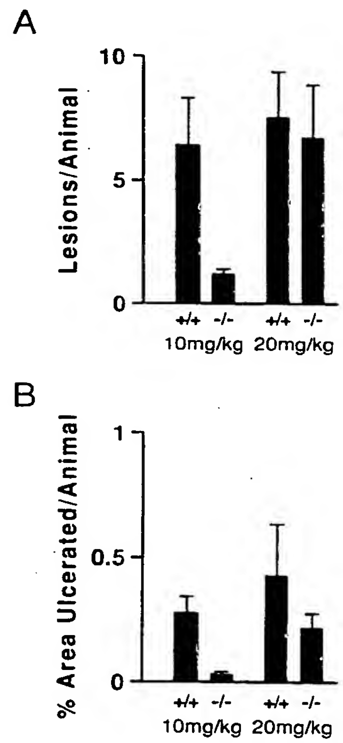

Figure 4. Induction of Stomach Ulceration by Indomethacin in Wild-Type and Homozygous Mutant Mice

(A) Data are expressed as the number of lesions of glandular stomach ulcers per animal at 10 and 20 mg/kg indomethacin. (B) Data are expressed as the percent ulcerated area of the total glandular stomach surface area at 10 and 20 mg/kg indomethacin. For (A) and (B), 10 mg/kg wild type (n = 9), homozygous mutant (n = 6); 20 mg/kg wild-type (n = 7), homozygous mutant (n = 8). Data are presented as the mean ± SEM.

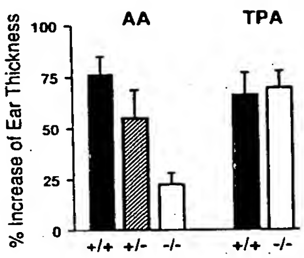

Figure 5. Induction of Ear Inflammation by AA and TPA

Data are expressed as the percent increase over pretreatment ear thickness. For AA and TPA treatments, five and four mice, respectively, of the F2 genotype indicated (wild type, heterozygous, homozygous mutant) were used. The mean control ear thickness was 0.22 ± 0.02 mm. Data are presented as the mean ± SEM.

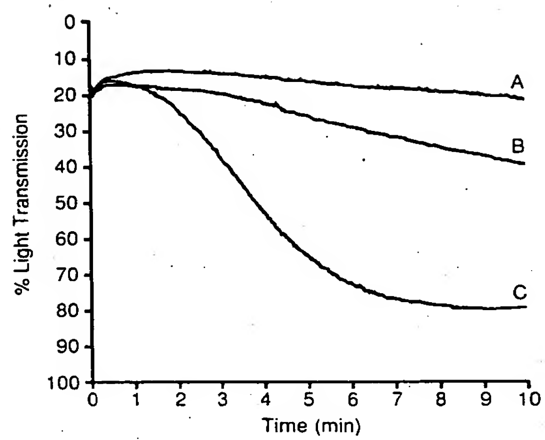

Figure 6. AA-Induced Aggregation of Platelets from Wild-Type F2 and Homozygous F2 Mutant Mice

(A) Solvent control with platelets from wild-type mice. (B) Platelets plus AA from homozygous mutant mice. (C) Platelets plus AA from wild-type mice.

0.36). At the 10 mg/kg dose, the homozygous mutant mice had statistically fewer lesions ($p < 0.04$), and a lower percent of stomach surface area was ulcerated ($p < 0.06$) than in the wild-type mice.

A possible means whereby gastric cytoprotection might be achieved in the COX-1 homozygous mutant mice is via a compensatory production of prostaglandins by the COX-2 isoform. To investigate this possibility, we determined PGE_2 levels in the glandular stomachs of COX-1 homozygous mutant mice and compared these levels with those in wild-type mice, untreated or after gavage with 40 mg/kg of indomethacin. Untreated wild-type levels were 113,430 ± 5,430 pg per milligram of tissue (n = 2); treated wild-type were 353 ± 126 pg per milligram of tissue (n = 2); homozygous mutant levels were 713 ± 265 pg per milligram of tissue (n = 2). Thus, it appears that COX-2 is contributing little to PGE_2 production in the stomach of COX-1 homozygous mutant mice.

Ear Inflammation

A standard ear swelling assay (Gad et al., 1986; Opas et al., 1985) was used to determine whether mice with the *Ptgs1* gene disruption had an altered inflammatory response to chemical challenge. The mice used in these studies were littermates from the F2 generation. Figure 5 shows that when AA was administered topically, the homozygous mutant mice had a significantly reduced ($p < 0.002$) inflammatory response (about 30% normal). The heterozygous mice also had a decreased response, although it did not reach statistical significance ($p < 0.26$). In contrast, inflammation in response to the potent tumor promoter tetradecanoyl phorbol acetate (TPA) did not differ in the wild-type and homozygous mutant mice. By 6 hr after treatment with AA, ear thickness had returned to normal, while TPA-treated ears remained inflamed for at least 18 hr after treatment.

In gene targeting experiments in which breeding is not confined to a single inbred strain of mice, the cosegregation of strain differences in genes linked to the target locus must be considered (Smithies and Maeda, 1995). In the present instance, this complication is effectively eliminated by the observations of Morham et al. (1995); their wild-type F2 *Ptgs2* animals, in which the wild-type *Ptgs1* locus and linked genes from the B6 and 129 strains can occur in all combinations, did not differ significantly in response to AA from our F2 wild-type animals carrying only the *Ptgs1* B6/B6 combination.

Platelet Aggregation

COX-1 is considered the key enzyme for generating prostaglandins involved in platelet aggregation (Funk et al., 1991). We therefore investigated the ability of platelets from wild-type and homozygous mutant mice to aggregate in vitro. The curves in Figure 6 show that platelets from homozygous mutant mice aggregate more slowly and to a lesser extent in response to AA than do platelets from wild-type mice.

Reproductive Capability of Homozygous Mutant Mice

Table 1 lists the frequency of live births and the litter sizes resulting from different heterozygous and homozygous matings. When homozygous mutant females are mated with homozygous mutant males, almost all of the pups are found dead, even though the litter sizes are normal. The cause of pup death has not been determined. In con-

Table 1. Effect of Parental Genotype on Litter Size and Pup Survival

Pairing: Male	Pairing: Female	Postnatal Survivors per Total Births[a]	Average Litter Size[a]
Homozygous ×	homozygous[b]	4 of 39	7.8 ± 1.2
Heterozygous ×	homozygous	20 of 26	6.5 ± 1.0
Homozygous ×	heterozygous	27 of 31	7.8 ± 0.5

[a] Data are based on four litters for the heterozygous × homozygous and five litters for the homozygous × homozygous pairs mated.
[b] Four different homozygous females, one of which produced two litters.

trast, when homozygous mutant females are mated with heterozygous males, the number of surviving pups and the litter sizes are close to normal, with the number of pups having the heterozygous and homozygous mutant genotypes being essentially equal. The breeding of homozygous mutant males to heterozygous females likewise results in normal pup survival and litter size. These data indicate that both homozygous mutant males and homozygous mutant females are fertile, but that pup survival is decreased when homozygous mutant mice are mated to each other; in this situation, neither the female nor the pups have functional COX-1.

Discussion

In the present study, we have used homologous recombination to disrupt the mouse *Ptgs1* gene that encodes COX-1. The major findings are that COX-1-deficient mice are generally healthy, do not have spontaneous stomach ulcers, and show less gastric ulceration than wild-type mice after gavage with indomethacin. The homozygous mutant mice also have a reduced inflammatory response to AA, and homozygous mutant × homozygous mutant matings result in reduced pup survival.

Because the stomach and kidney are two tissues where COX activity had been thought essential for proper function (Robert, 1975, 1979; Clive and Stoff, 1984; Black, 1986; Brooks and Day, 1991; Price and Fletcher, 1990; Simon, 1994), the lack of pathology in these tissues of COX-1-deficient mice was surprising. Furthermore, based on the hypotheses that COX-1 is a housekeeping enzyme and that NSAID inhibition of COX-1 is responsible for stomach ulceration, we had expected that COX-1 deficiency might lead to spontaneous stomach ulceration or bleeding in the homozygous mutant mouse. But the gastrointestinal tissues of the homozygous mutants were not distinguishable from wild type. Thus, absence of COX-1 is not sufficient to cause stomach ulceration in mice. Furthermore, measurement of PGE_2 in the glandular stomach of COX-1 homozygous mutant mice indicates that PGE_2 levels per milligram of tissue are less than 1% of the levels observed in wild-type mice. This reduction is consistent with that observed in peritoneal macrophages from homozygous mutant mice (Figure 3) and is approximately equal to the levels observed in the stomachs of wild-type mice treated with 40 mg/kg indomethacin. The low level of PGE_2 in the glandular stomach of the homozygous mutant mice coupled with the finding that COX-2 is undetectable by Western blot analysis (data not shown) suggests that compensation by COX-2 is not a significant factor in this tissue in COX-1 homozygous mutant mice.

Because of the absence of spontaneous gastric ulceration in the COX-1-deficient mice, we determined whether they have an altered sensitivity to NSAID-induced gastric ulceration. For this we chose indomethacin, which is widely used in NSAID studies of gastric ulceration and is known to induce ulceration in the mouse stomach (Yokoyama et al., 1985; Rainsford, 1987; Ettarh and Carr, 1993). Our experiments confirm that wild-type mice are sensitive to stomach ulceration by indomethacin gavaged at either 10 or 20 mg/kg, doses commonly used to cause ulceration in rats (Futaki et al., 1993; Rainsford, 1993; Beck et al., 1990). Surprisingly, we found that the homozygous *Ptgs1* homozygous mutants are less sensitive to indomethacin-induced stomach ulceration than are the wild-type mice: the absence of COX-1 decreases rather than increases the incidence of ulceration after indomethacin treatment. Thus, these observations suggest that indomethacin-induced gastric ulceration may be due to mechanisms other than (or in addition to) COX-1 inhibition. Alternatively, in the development of gastric ulcers, lack of COX-1 activity due to gene disruption may not be equivalent to the inhibition of COX-1 activity by indomethacin. As described by Morham et al. (1995), mice deficient in COX-2 also show no spontaneous stomach ulcers or overt intestinal lesions. These data emphasize that the relationship between inhibition of COX activity and ulceration is complex, and they illustrate that the COX-1- and COX-2-deficient mice provide novel ways of studying isoform-specific NSAIDs and for identifying mechanisms in addition to COX inhibition that may be involved in the ulcerative process.

The kidneys of the COX-1-deficient mice showed only minimal abnormalities even at 5 months of age. Therefore, absence of COX-1 in the kidney is not deleterious under normal physiological conditions.

Prostaglandins have key functions in various stages of the reproductive process, ranging from ovulation and spermatogenesis to parturition (Thorburn, 1991, 1992; Zahradnik et al., 1992). Which COX isoform is involved in each of these stages is not known, except for ovulation, when it appears that COX-2 is the important form (Sirois et al., 1992). Our studies show that neither male nor famale fertility appears to be affected by lack of COX-1 (Table 1), but they clearly show that complete lack of COX-1, such as occurs in homozygous mutant × homozygous mutant matings, severely impedes survival of pups perinatally. Because prostaglandins are known to be involved in the initiation of labor (Kelly, 1994), it may be the onset of labor that is impaired. However, the normal litter size and pup survival seen when heterozygous males are mated with homozygous mutant females show that absence of COX-1 synthesis in the mother can be overcome by the presence of COX-1 in as few as 50% of the pups or in their placental material. From these matings, heterozygous and homozygous mutant pups are born in about equal numbers. The mating of homozygous mutant males with heterozygous females likewise results in normal litter sizes and pup survival. The most likely explanation for these observations is that prostaglandins from the COX-1 pathway in either the maternal or fetal tissues are essential for normal parturition. Further studies are needed to elucidate the roles of the COX isoforms in pregnancy and parturition, and the COX-deficient mice should provide useful models for such studies.

The decrease in AA-induced platelet aggregation seen in our COX-1-deficient mice accords well with existing data that platelets contain the constitutively produced isoform

COX-1 (Funk et al., 1991). Since platelets lack nuclei, this deficiency cannot be overcome by any compensatory induction of *Ptgs2* transcription followed by COX-2 synthesis.

The COX-1-deficient mice clearly have a reduction in their responses to AA, although their responses to TPA do not differ from wild type. However, the COX-2-deficient animals are as sensitive to inflammation caused by both AA and TPA as are wild-type mice. A possible reason for the differences between AA and TPA effects in the COX-1-deficient mice is that AA in wild-type mice (and COX-2-deficient mice) can be immediately metabolized by the constitutive COX-1 enzyme to PGH_2 and subsequently to PGE_2, which contributes to edema and inflammation. Since this cannot occur in the COX-1 homozygous mutant mice, less inflammation occurs. TPA, on the other hand, does not interact directly with COX-1 but is known to induce the synthesis of other enzymes, including COX-2 in vitro (Kujubu et al., 1991; DuBois et al., 1994a) and in vivo (Muller-Decker et al., 1995). The finding that TPA-induced inflammation is equal in COX-2 homozygous mutant mice and wild-type mice indicates that COX-2 is not essential for this type of inflammation to occur in the skin of these mice. Thus, it appears that COX-1 can contribute to inflammation. More detailed studies of the responses of the COX-1- or COX-2-deficient mice will be needed to indicate the relative roles of the two isoforms following different types of inflammatory stimuli.

The major conclusions from the present study are that lack of COX-1 does not cause spontaneous gastric ulceration and decreases indomethacin-induced ulceration, that it decreases inflammatory responses to AA, that it decreases platelet aggregation, but that it has no other overt systemic effects except those associated with parturition. Lack of COX-2, as reported by Morham et al. (1995), also does not cause spontaneous stomach ulceration, but, in contrast with lack of COX-1, it has no effect on inflammatory responses to AA, causes severe kidney disease, and leads to spontaneous peritonitis in some animals.

Experimental Procedures

Targeting Vector Construction

A Charon 35 genomic library containing DNA from E14TG2a mouse ES cells (Hooper et al., 1987) was screened with a 357 bp probe (see below) for the 5′ end of exon 11 of the mouse *Ptgs1* gene. A clone containing approximately 15 kb of the 3′ end of the *Ptgs1* gene was isolated, and overlapping 6 kb XbaI and ClaI fragments (Figure 1A) were subcloned into Bluescript. A 4.3 kb NotI-XhoI fragment of the XbaI subclone was inserted into the pPNT vector (Tybulewicz et al., 1991) 5′ to the *Neo* gene, and a 2.3 kb BamHI fragment from the ClaI subclone was inserted 3′ to the *Neo* gene to produce the targeting construct (Figure 1B).

Cell Culture and Targeting

E14TG2a ES cells were cultured by conventional methods on feeder cells. The targeting vector was linearized with NotI and electroporated at 5 nM into about 10^8 trypsinized ES cells using a 1 s pulse at 300 V and 200 µF. Positive/negative selection was performed with G418 and ganciclovir (Mansour et al., 1988) with ganciclovir providing about a 10-fold enrichment over G418 alone. Ganciclovir- and G418-resistant colonies were isolated 8–9 days after electroporation and transferred individually to 24-well plates previously seeded with feeder cells. The cells were trypsinized 2 days later and reseeded into 6-well plates. After 2 days the cells were trypsinized, and about half were frozen at −80°C. DNA isolated from the remaining half was used for PCR and genomic Southern blot analysis.

PCR and Southern Blot Analysis

PCR was the initial screen for identifying targeted ES cells and mice carrying the disrupted *COX-1* gene (Kim and Smithies, 1988). A primer specific for the *Neo* gene and a second primer specific for a genomic sequence 3′ to sequences in the targeting construct (Figure 1C) produced a single 2.4 kb band diagnostic of targeting. Genomic Southern blots were produced by standard techniques and probed with a random primer ^{32}P-labeled probe (Stratagene Prime-It II) made from the 357 bp PCR fragment specific for the 5′ region of exon 11. DNA was isolated from mouse tails to determine genotype.

Northern Blot Analysis

Total RNA was isolated from tissues by homogenizing them in TRIzol (Life Technologies) or from cells by scraping directly into TRIzol as recommended by the supplier. Each sample (15 µg) was electrophoresed in a 2.2 M formaldehyde–0.9% agarose gel. After capillary transfer, the blot was hybridized with a random primer ^{32}P-labeled probe made from the exon 11–specific 357 bp PCR fragment or a 1.7 kb *COX-1* cDNA fragment (Oxford Biomedical Research Corporation). Blots were stripped and probed for actin to ensure equal loading of RNA.

Western Blot Analysis

To prepare microsomes, we homogenized tissues in buffer (0.1 M Tris-HCl [pH 7.4], 2 mM EDTA, 10 mg/ml leupeptin, 20 mg/ml aprotinin, 0.5 mM phenylmethylsulfonyl fluoride) and then sonicated them at 30% power (Fisher Scientific, Model F50) three times for 15 s. The homogenates were centrifuged at 10,000 × g for 15 min at 4°C. The resulting supernatants were centrifuged at 100,000 × g for 1 hr at 4°C, and the microsomal pellets were sheared in buffer (100 mM Tris [pH 6.8], 8% SDS, 20% glycerol) with a 25-gauge needle. An aliquot was removed for protein determination (Bio-Rad DC) before boiling with bromophenol blue (0.05% [w/v]) and 2-mercaptoethanol (6% [v/v]).

For immunoblot analysis, 40 µg of microsomal protein (kidney and stomach), 20 µg of microsomal protein (colon), or 10 µg of protein from cell lysate (macrophages) were separated by SDS-PAGE using the Mini-PROTEAN II electrophoretic apparatus (Bio-Rad). Proteins were transferred onto Hybond–ECL nitrocellulose (Amersham) using the Mini Trans-Blot electrophoretic transfer cell system (Bio-Rad). Membranes were blocked in 5% nonfat milk–Tris-buffered saline with 0.1% Tween 20 (TBST) before incubating with a rabbit antibody to murine COX-1 provided by Dr. D. DeWitt (Morita et al., 1995) or to COX-2 (Cayman Chemical). Blots were incubated with anti-rabbit IgG horseradish peroxidase–linked secondary antibody (Boehringer Mannheim) in TBST and 1% nonfat milk. Chemiluminescent detection was performed using reagents from Amersham, and bands were visualized after exposure to Hyperfilm–ECL (Amersham).

Indomethacin-Induced Stomach Ulceration

Indomethacin was suspended in 1% methyl cellulose at the concentration of 1 or 2 mg/ml and given at the stated doses by gavage. All animals were fasted 16–18 hr prior to gavage. Animals were euthanized using CO_2 6 hr after treatment, and their stomaches were removed and opened along the lesser curvature. Stomach lesions were scored as described by Ghanayem et al. (1987). The number of lesions were counted, an enlarged image of the formalin-fixed glandular stomach and of each individual lesion was traced, and the area of each lesion was determined using a computer-assisted image analysis system. The total area of the stomach was traced and measured. The area of all lesions in each stomach was calculated and divided by the area of the glandular stomach to derive the percent of area with lesions. All samples were scored blind. No ulceration was shown by eight wild-type mice that received vehicle alone.

Mouse Ear Inflammation Assay

AA (2 mg per 10 µl) or TPA (1 µg per 10 µl) in acetone was applied

to the inside of the left ear and 10 μl of acetone was applied to the right ear as described by Opas et al. (1985). Ear swelling was determined after 2 hr for AA or 6 hr for TPA by the method of Gad et al. (1986).

Macrophage Isolation and PGE_2 Analysis

Peritoneal macrophages were isolated and LPS stimulated by modification of the procedure of Watanabe et al. (1994). Thioglycolate-elicited macrophages were isolated by peritoneal lavage with 5 ml of cold RPMI 1640 medium. Macrophages were seeded at 1×10^7 to 2.5×10^7 cells per 60 mm dish, depending on yield, and allowed to attach for 2 hr in a humidified incubator with 5% CO_2 in air. The plates were then washed with Hank's balanced salt solution to remove nonadhering cells, and medium containing 1% serum with or without LPS (10 μg/ml) was then added. Attached cell numbers were determined by counting with an eyepiece micrometer. After a 6 hr incubation, the medium was removed and replaced for 30 min with medium containing 10 μM AA. Subsequent analysis for PGE_2 in the medium was by a competitive radioimmunoassay (Amersham).

Platelet Aggregation

Platelet aggregation was carried out as described by Paigen et al. (1987) using AA to induce aggregation. For each assay, blood was pooled from two mice (about 1 ml in total volume) and centrifuged to prepare platelet-rich and then platelet-poor plasma. The assay was conducted with 300 μl of plasma at 3×10^8 platelets per milliliter. Aggregation was induced by adding 15 μl of Na–AA (22 mg/ml in Na_2CO_3 buffer). Turbidity was measured with a Lumi-Aggregometer.

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